THE STABILITY OF CAROTENE IN PLANT TISSUES [1]

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ONE FIGURE

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The importance of vitamin A in animal nutrition is quite generally recognized, and many references may be found in the literature giving the vitamin A potencies of various foods and feeding stuffs. Comparatively little work has been done, however, on the stability of the vitamin under the usual conditions of storage. Since it is nearly always necessary to store feeds from one growing season to the next or, in the case of experimental work, for an extended assay period, a knowledge of the stability of the vitamin in plant materials should be of value. The vitamin A activity of plant tissue may be largely, if not wholly, ascribed to the carotene content and, therefore, these studies were mainly carried out by means of colorimetric carotene determinations. In certain cases the results of these determinations were compared with the results of bio-assays.

REVIEW OF LITERATURE

The fact that the vitamin A potency of a plant material may be ascribed to its carotene content has been demonstrated by several workers. Euler, Demole, Karrer and Walker ('30)

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were among the first to point out this fact. Similar observations have been made in this laboratory (Russell, Taylor and Chichester, '32, '34) and by Guilbert ('34).

In the majority of the studies which have been conducted on the stability of carotene and vitamin A during storage, the conditions have been similar to those in common practice, namely, in bulk or in sacks in a barn or in containers in the laboratory exposed to light and air. Under these conditions the results have been somewhat variable. Thus Roberts ('31) found no loss of the vitamin A in yellow corn when it was spread on the floor of the laboratory for 6 months. Bethke and Kick ('29) reported little loss of vitamin A during a year's storage of alfalfa meal or yellow corn. These workers also found that the degree of fineness of the yellow corn had no effect on the stability of the factor. Russell, Taylor and Chichester ('34) found no loss of carotene during 2 to 3 months storage of finely ground alfalfa when the material was stored in vacuo at $0° \pm 5°$C. On the other hand, Fraps and Treichler ('33) found that alfalfa leaf meal lost 50% of its vitamin A in 11 months, and yellow corn 30 to 50% in 6 months. Miller and Bearse ('34) reported some loss of vitamin A when dehydrated grass, dehydrated alfalfa, and sun-cured alfalfa were stored for 1 year, the rate of loss being greater with the grass than with the alfalfa. Guilbert ('35) found that a considerable loss of carotene content might occur in stored alfalfa, the loss being much more rapid at elevated temperatures. Similar findings have been reported by Woodward ('36) and by Wiseman, Kane and Carey ('36). The latter investigators noted that the degree of fineness had very little effect. According to Shepherd, Woodward and Graves ('36) and Woodward ('36), the loss of carotene was very nearly complete when hay of a high moisture content was stored.

The storage of forage crops as silage has become increasingly popular in recent years. A recent method which is being used fairly widely, particularly for the ensiling of leguminous crops, entails the addition of mineral acids to the green crop

and is known as the A.I.V. process, having been named from the initials of the inventor, A. I. Virtanen. In this process the addition of mineral acids lowers the pH of the mixture to a point where little fermentation can occur. Virtanen ('34), in a summation of his work on the A.I.V. process, reports that it is very efficient in preserving most of the original nutrients of the green crop, including the carotene. Peterson et al. ('35) found a higher carotene content in silage made from alfalfa or from soy bean plants by the A.I.V. process than was present in the fresh green plants. They ascribed this apparent increase in carotene to the effect of the acid in rendering the carotene more readily extractable. Similar findings have been reported by Krauss and Washburn ('36). However, according to Kane, Wiseman, Hartman and Cary ('36), spectrophotometric data indicate the presence in the extracts from acid treated silage of an unidentified pigment, probably formed by the action of acid on xanthophyll, in such large amounts as to render a correct determination of carotene impossible. A smaller amount of this interfering pigment was also found in ordinary corn silage to which no mineral acids had been added. This work raises some question as to the value of carotene determinations on silages. Karrer, Euler and Solmssen ('34) have shown that some, but not all of oxides of carotene may possess vitamin A activity depending upon whether the ionone ring has been broken. Possibly some oxidative change may account for any discrepancy between carotene analyses and the biological assay.

PROCEDURE

Artificially dried alfalfa hay was obtained from the Walker-Gordon Laboratories, Inc., in August, 1933. Eleven bags containing approximately 40 pounds each of coarsely chopped alfalfa, were taken consecutively from the drier. Ten of these were stored immediately in a barn and the remaining bag, which was the sixth one of the series of eleven, was taken to the laboratory for experimental studies. This latter bag was sampled carefully and a portion chopped in a Wiley

mill until it passed a 1 mm. screen. A portion of the Wiley-milled material was further ground in a pebble mill to a very fine powder. Samples were then stored under the following conditions.

Sample 74 Coarsely chopped, stored in barn
Sample 75 Wiley-milled, in sealed bottle, in dark, room temperature
Sample 76 Wiley-milled, in sealed bottle, in dark, 0° ± 5°C.
Sample 77 Ball-milled, in vacuo, in dark, 0° ± 5°C.
Sample 78 Wiley-milled, in vacuo, in dark, 0° ± 5°C.
Sample 79 Ball-milled, in sealed bottle, in dark, room temperature

At the beginning (August, 1933) and at intervals thereafter up to March, 1935, carotene determinations were made on these samples, using the method of Russell, Taylor and Chichester ('35). A biological assay for vitamin A potency was run on samples 74 and 77 after 19 months of storage.

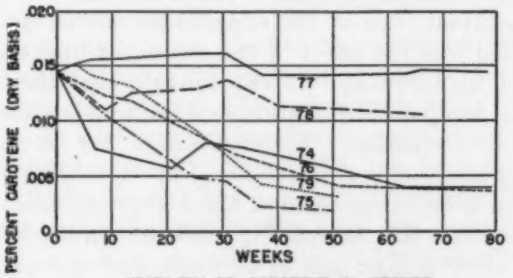
Somewhat similar studies were carried out on ordinary corn silage which had been preserved by allowing the usual fermentation process to occur and on corn and on alfalfa which had been ensiled without fermentation by means of the addition of mineral acids (A.I.V. process). The samples analyzed for carotene were obtained at random from large silos. It was, therefore, impossible to obtain samples of identical origin. The wet material was ground through a motor driven meat chopper and stored in tightly stoppered jars at 0° ± 5°C. until analyzed. One sample of ordinary corn silage was assayed biologically for vitamin A potency.

EXPERIMENTAL RESULTS

Studies with alfalfa hay. The results of the carotene determinations are shown graphically in figure 1. As may be seen, the two samples which were kept in vacuo at 0° ± 5°C. have shown a high degree of stability with regard to their carotene contents. This was particularly true in the case of sample 77 which was in the ball-milled condition. Considering all the values obtained during the 20-month storage period, the coefficient of variation for this material was only 5.0%, whereas the curve for the Wiley-milled sample, no. 78, exhibited more variation and a definitely lower trend. The loss of carotene in sample 78 was of the order of 25% in 20 months.

Possibly this loss was caused by the fact that, prior to storage, the Wiley-milled material was exposed to the atmosphere of the laboratory for several days longer than the finely-ground sample. Whatever the cause, the difference was further demonstrated by samples 75 and 79 in which no. 79, the ball-milled specimen, was again significantly higher than no. 75, Wiley-milled, even though the two were stored under identical conditions.

Because of the difference in the behavior of the ball-milled and the Wiley-milled materials, comparisons of the various



STABILITY OF CAROTENE IN STORED ALFALFA HAY.

Fig. 1 No. 74, coarsely chopped, stored in barn. No. 75, Wiley-milled, in sealed bottle in the dark at room temperature No. 76, Wiley-milled, in sealed bottle in the dark at 0° ± 5°C. No. 78, Wiley-milled, in vacuo in the dark at 0° ± 5°C. No. 77, Ball-milled, in vacuo in the dark at 0° ± 5°C. No. 79, Ball-milled, in sealed bottle in the dark at room temperature.

methods of storage are reliable only when made on similarly ground materials. Hence, samples 77 and 79 are placed in one group and samples 75, 76 and 78 in the other. As previously noted, the carotene content of sample 77, which was ball-milled and stored in vacuo in the dark at 0° ± 5°C., showed little change during 20 months of storage. In contrast, sample 79, which was likewise ball-milled and stored in a sealed but not evacuated bottle in the dark at room temperature, was found to have lost 77% of its carotene after 12 months. This loss is ascribed to the combined effect of the presence of air and of the higher temperature.

The other group of samples, nos. 75, 76 and 78, were stored in the Wiley-milled condition. Of these samples, no. 78, which was in vacuo in the dark at 0° ± 5°C., showed the least change in carotene, the loss being 23% in 12 months. Sample 76, stored in a sealed but not evacuated bottle in the dark at 0° ± 5°C., lost 70% of its carotene in the same period while sample 75, which had been stored under identical conditions, except that the temperature was that of the laboratory, lost 87%. From these data it appears that both the exclusion of air and low temperature are important factors in the preservation of the carotene content of dried alfalfa.

In the original plan of the experiment one of the factors to be studied was the effect of the degree of fineness on the stability of the carotene. It was thought that the carotene in the more finely divided material might be more susceptible to destructive changes. Experimentally the reverse was found to be the case but, as indicated previously, the reasons are believed to be independent of the degree of fineness. This fact, and the fact that sample 77, which was in the ball-milled condition, showed little loss of carotene during long storage, indicate that the degree of fineness has relatively little effect on the stability of the carotene.

The results on sample 74 are of practical interest in that this sample was kept under conditions identical with those which were being employed in the storing of large quantities of alfalfa hay. Hence, the results may be interpreted to apply generally to stored hays. Difficulties in sampling such coarsely divided material make the results somewhat variable. However, the data show that approximately 50% of the original carotene content was lost during the first 3 months (August, September, October) of storage. There was only a slight loss during the succeeding winter months, but an additional loss of 25% occurred during the spring, summer and fall months so that the carotene content was reduced to one-fourth of its original value. No further loss occurred during the second winter of storage. These results are further proof that temperature has an effect on the rate of destruction of

carotene in stored hays and agrees with the results reported by Guilbert ('35), Woodward ('36) and Wiseman, Kane and Cary ('36). The results also point out the danger of relying on alfalfa hay, which has been stored for some time, as a particularly rich source of vitamin A for domestic animals. As will be indicated later in this paper, material such as silage, in which the carotene appears to be fairly stable, may be an equally good source of this vitamin.

The results on alfalfa samples 77 and 78 show that limited quantities of such materials may be preserved for experimental purposes so that there is little loss of carotene during long periods of storage.

In order to determine whether the carotene content, as measured colorimetrically, was still an index of vitamin A potency after long storage, bio-assays were made on alfalfas 74 and 77. The method employed was essentially that of the U. S. Pharmacopeia XI. Sample 74 had been stored in a barn for 19 months and had lost approximately 75% of its original carotene content at the time the assay was run. Sample 77 had been stored in the ball-milled condition in vacuo in the dark at $0° \pm 5°$C. for the same period of time and retained the greater portion of the original carotene. These two samples were chosen because of the wide difference in their degree of carotene preservation. It was thought that, if a change had occurred which would make the carotene determination less reliable as an index of vitamin A potency, this change might be more apparent in a sample such as no. 74.

The results of the bio-assays are shown in table 1, in which the vitamin A values were estimated by comparing the growth responses of the test groups of rats with a growth response-dosage curve, obtained by feeding several levels of the U.S.P. reference cod liver oil. At the 1.0 microgram feeding level, alfalfa 74, the barn stored material, which had lost approximately 75% of its carotene during 19 months' storage, gave a decidedly lower growth response than did alfalfa 77, which had not lost carotene during storage. At the 2.0 microgram feeding level, alfalfa 74 was also lower than no. 77, though

the difference was not as marked. However, since the growth response curve tends to flatten out at the higher feeding levels, this behavior was to be expected. The fact that the experimental animals showed a dislike for alfalfa 74 may, in part, account for the lower growth response on this supplement. However, the difference between the responses to the two samples, particularly on the 1.0 microgram feeding level which is believed to be the more sensitive, is large enough so that a real difference probably exists. It appears, therefore,

TABLE 1

Biological assays on alfalfas and silage

SUPPLEMENT	STORAGE PERIOD	CAROTENE CONTENT	GROWTH RESPONSE	VITAMIN A[1] CONTENT OF SUPPLEMENT	VITAMIN A[2] PER 0.6 MICROGRAM OF CAROTENE
	months	*microgram*	*gm.*	*U.S.P. units*	*U.S.P. units*
25 mg. alfalfa 74	19	1.0	14.6 ± 2.1	2.10	1.26
50 mg. alfalfa 74	19	2.0	44.0 ± 3.7	4.00	1.20
Average alfalfa 74					1.23
7.5 mg. alfalfa 77	19	1.0	34.0 ± 3.0	3.23	1.93
15.0 mg. alfalfa 77	19	2.0	55.0 ± 4.5	5.44	1.63
Average alfalfa 77					1.78
50 mg. corn silage 3	6	0.7	23.7 ± 2.9	2.55	2.19
100 mg. corn silage 3	6	1.4	43.3 ± 35	3.95	1.69

[1] Values obtained by comparison with a growth response-dosage curve of rats for the U.S.P. reference cod liver oil.

[2] Calculated values. Six-tenths microgram of carotene was chosen since it is the international unit of vitamin A.

that the destruction of carotene in sample 74 was accompanied by a change in the remaining carotene which caused it to have a lower vitamin A potency per unit of carotene. Possibly this behavior may be ascribed to the formation of a certain amount of an oxide of carotene but no data were obtained which might indicate the nature of the change.

Studies with silage. Table 2 shows the results of the carotene determinations on silage as well as the values for fresh corn plants, fresh alfalfa and artificially dried alfalfa. Many of the individual samples were not closely related in that

they were taken from different fields or silos and at different times. Also, considerable variation was found between duplicate determinations, particularly in the case of the fresh crops and the silage, due largely to the difficulty of sampling the heterogeneous material. However, the results are believed to be a fairly reliable index of the amounts of carotene which may be expected to be present in these materials.

The results in table 2 show that there was no loss of carotene during the curing processes; in fact there was apparently some increase. Although this increase was probably more apparent than real, nevertheless several possible explanations may be advanced, particularly in the case of the silage.

TABLE 2

Carotene contents of silage

Kind of material	*Number of samples*	*Carotene (dry basis), %*
Fresh alfalfa	5	0.0176
Machine dried alfalfa	6	0.0195
A.I.V. alfalfa silage	4	0.0215
Fresh corn plant	10	0.0050
Regular corn silage	8	0.0060
A.I.V. corn silage	4	0.0070

For example, assuming no loss of carotene, the fermentation process would cause the formation of volatile products, decreasing the amount of solid constituents but increasing the relative amounts of the non-fermentable materials of which carotene is one. The loss of water-soluble constituents by drainage would also have some concentrating effect on the carotene. Furthermore, as has been pointed out earlier in this paper, the action of the acid (Kane, Wiseman, Hartman and Cary, '36) or of atmospheric oxidation (Karrer, Euler and Solmssen, '34) may give a high color value to the carotene fraction as obtained in the colorimetric determination, not all of which is due to carotene.

The data of table 2 should not be interpreted to indicate that the A.I.V. process is more efficient in the preservation of carotene than the ordinary fermentative method. The

results in table 2 are average values obtained on miscellaneous samples and are intended to show the general level of carotene in the crops analyzed, rather than a strict comparison between the various types of materials. Furthermore, the difference between the value for regular corn silage, 0.006%, and that for A.I.V. corn silage, 0.007%, is of doubtful significance. However, the data do indicate that the carotene in various A.I.V. silages is very stable. Table 3 shows values obtained on two types of this silage, and it may be seen that practically no change occurred during 5 months of storage. From these results it would appear that this material is a more reliable source of carotene than alfalfa hay, since the silage did not

TABLE 3

Stability of carotene in silage

Material	*Storage period, months*	*Carotene (dry basis), %*
A.I.V. corn silage	2	0.0086
A.I.V. corn silage	3	0.0063
A.I.V. corn silage	4	0.0060
A.I.V. corn silage	5	0.0067
A.I.V. alfalfa silage	3	0.0218
A.I.V. alfalfa silage	4	0.0218
A.I.V. alfalfa silage	4	0.0221
A.I.V. alfalfa silage	5	0.0203

exhibit the original rapid loss which occurred with the barn stored hay.

As a check on the carotene determinations a biological assay was conducted on one sample of regular corn silage. Unsuccessful attempts were made to preserve the silage during the test period by drying in vacuo over sulfuric acid and then storing in vacuo at 0°C. As shown in table 4, it was found that a rapid destruction of carotene took place both during the drying process and during the subsequent storage. It is possible that concentration of various acids which occurred in the drying process may account for this destruction. However, no further trials of this nature were made since it was found that the material could be preserved readily in the refrigerator in the wet state, in tightly stoppered bottles and

that in this condition it was readily eaten by the rats. The results of the biological assay, table 1, show a high growth response compared with the amount of carotene present, similar to that obtained with alfalfa 77. This indicates that, in the case of the regularly fermented corn silage, no change has occurred in the carotene which would make a carotene determination unreliable as an index of vitamin A potency.

TABLE 4

Loss of carotene during drying of corn silage

Treatment	*Carotene (dry basis), %*
Before drying	0.0064
Immediately after drying	0.0033
1 week after drying	0.0021
5 weeks after drying	00016
7 weeks after drying	0.0009

SUMMARY

1. Chopped, artificially dried alfalfa hay, stored in bags in a barn, lost 50% of its carotene during the first 3 months of storage (late summer and early fall). There was no loss of carotene during the following winter but a further loss of 25% occurred during the succeeding summer season. No loss occurred during the second winter of storage.

2. Storage of ball-milled, artificially dried alfalfa hay in vacuo in the dark at 0° ± 5°C. preserved the carotene very efficiently for 20 months. Both the exclusion of air and low temperature were important factors in the carotene preservation. The degree of fineness was of little importance.

3. A.I.V. corn silage lost approximately 20% of its original carotene content during 5 months storage. In the case of A.I.V. alfalfa silage there was practically no loss in 5 months.

4. Regular corn silage, during drying in vacuo over sulfuric acid, and storage in vacuo in the dark at 0° ± 5°C. lost carotene very rapidly.

5. Biological assays on the hay stored in vacuo, and on a sample of regular corn silage, showed that the carotene content of these materials was a good index of their vitamin A

potencies. In the case of the barn stored hay, the bio-assay gave relatively lower values. The data suggest that, as the carotene was destroyed during storage, a change also occurred in the remaining carotene which rendered it less potent biologically.

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DIGESTIBILITY AND NUTRITIONAL VALUE OF CEREAL PROTEINS IN THE HUMAN SUBJECT[1]

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Several years ago the writers undertook to apply the Thomas ('09) biological-value method with improvements to a study of cereal proteins on six human subjects. The results, we are convinced, were more reliable than those of Thomas, because the periods were longer, and perhaps equal to those of Martin and Robison. They were published only in preliminary form because of the conviction that the method of obtaining metabolic nitrogen[2] would have to be improved still further or an alternative method adopted. The chief difficulty lay in the unphysiological character of the basal (protein-free) diet which was adopted, consisting of cornstarch, cane sugar, milk sugar, cream and fruit juices. There were frequent watery stools[3] caused by excessive carbo-

[1] This paper was to have been one of the series on cereal breakfast foods begun in this journal in 1929. It has been delayed because some of the data lacked confirmation, which it has been possible to supply only recently, and which will be published soon. Preliminary reports of the results were made, Proc. Soc. Exp. Biol. and Med., 1926, vol. xxiii, p. 458 and Proc. XIIth Internat'l Congr. of Physiologists, Stockholm, Skand. Arch. f. Physiol., 1926. This article has not been given a serial number because the present editorial policy excludes this practice.

[2] The term 'metabolic nitrogen' is objectionable for the reason that it is not truly metabolic in the sense implied. A more accurate generic form is 'alimentary nitrogen,' or, in full, alimentary contribution to the fecal nitrogen. The shorter term will be used in this and subsequent papers, reserving metabolic nitrogen for the catabolic products excreted in the urine.

[3] Thomas' notes on the character of the feces in his low protein periods contain frequent references to 'wässerig' stools.

hydrate fermentation, as was true, and doubtless for the same cause, in the original experiments of Thomas. One subject was obliged to discontinue the experiment after the first basal period, another was nauseated in three of the seven periods so that he was unable to eat the entire diet, and milder degrees of anoerxia were experienced by all. An attempt to improve the method by introduction of fruits with low nitrogen content instead of fruit juices into the basal diet as sources of bulk and vitamins, also vitamin B complex concentrate, was only partially successful. Biological values were calculated because there were several good 'non-protein' diet periods on the five remaining subjects in spite of the loss of several periods. The cereal nitrogen plus cream and other nitrogen was fed at the level of the nitrogen losses on 'non-protein' basal diets, and probably for this reason the alimentary disturbances were not wholly confined to the basal periods. The diet was still too largely carbohydrate for the human alimentary tract. For these reasons the results will not be given in detail. The biological values will be introduced for comparison with others later in this paper.

The next year another study of the nutritive value of cereal proteins was undertaken but by a different procedure. The earlier experience led to the conviction that a proper application to human subjects of the strict biological value method as worked out by Mitchell ('24) for rats would always entail such hardship as to jeopardize the results.[4] Martin and Robison ('22) enumerate six 'factors' which must be considered if valid results are to be obtained. They may be described briefly as follows: 1) adequate length of time on a constant intake (of non-protein diet); 2) adequate length of period (experimental) to eliminate errors due to fluctuation of N-output; 3) the necessity for abundant energy supply; 4) reduction of nitrogen in the basal diet to a 'minimum'; 5) adequate vitamins and salts; 6) the difficulty of correctly apportioning the nitrogen of the feces (to food residue and alimentary excretion).

[4] A later attempt in this laboratory (Sumner and Murlin, '38) succeeded with only four out of ten subjects tried.

Anxious to overcome the obvious defects of Thomas' experiments these authors prolonged the basal periods (done in a different season of the year from their protein periods) apparently to the limit of endurance in order to reach the minimal N-output in the urine for the particular non-protein basal diets. In their protein periods they ingested varying amounts of the protein, thereby obtaining variable biological values, and they did not wholly succeed in keeping calory intake constant in basal and experimental periods. They calculated their values in two ways: first, assuming that all the nitrogen of the feces came from the body (Thomas' formula B) and second, assuming that the amount by which the N of the feces on protein exceeds the amount so excreted on N-free diet represents the unabsorbed food. They admit the fallacy of the second assumption in their statement that "the truth will probably lie somewhere between the two extremes."

While this work without doubt represents a considerable improvement over the experiments of Thomas, it falls far short of the rigid control possible with rats. Boas-Fixsen ('35) has summarized the refinements of control which have been gradually introduced by investigators using these animals as follows: 1) constancy of non-nitrogenous components of the diet; 2) adequate vitamins and salts; 3) long pre-experimental adjustment period; 4) experimental period as long as practicable; 5) use of a small amount of high value protein in endogenous (basal) periods (introduced by Mitchell and Carman, '26); 6) frequently repeated endogenous periods; 7) animals of similar weight, because of the relation of endogenous N to body weight; 8) equal roughage because of its effect on endogenous fecal N; 9) feeding different proteins at the same level. The first four items of this list cover essentially the first 5 of the list of Martin and Robison. Items 5 to 9 are new.

All students of nutrition with experience in N-balance experiments will agree that all of these controls are important, and the introduction by Mitchell and Carman of a small

amount of high-value protein in the control or basal period tends to correct the unphysiological character of the previously used non-protein diet but does not go far enough. The alimentary tract cannot be supposed to respond in like manner to a diet containing in alternate periods no protein and sufficient protein to balance the endogenous losses. It is known that protein in direct contact with the mucosa is one of the best stimulants to gastric and intestinal secretions, the residues from which constitute the bulk of 'normal' feces. Convinced that this qualitative effect on secretion cannot be ignored, the writers have withheld this paper from publication for several years until confirmatory evidence of a more positive character was at hand. The evidence referred to is contained in the paper recently published from this laboratory by Bourns, Nasset and Hettig ('36). This report makes clear that, in the dog, protein (beef heart) taken alone stimulates secretion by the newly discovered humoral mechanism controlling the intestinal gland, more than does starch (potato) or cream. But when added to a basal diet containing a fair quantity of protein, the starch and cream stimulate more than extra protein. The solids in the secretion do not change much; hence greater volume means greater potential feces production. In view of these facts the assumptions underlying the biological value method require revision.

In setting up the revised experimental procedure the writers resolved to eliminate so far as possible the inequalities of the control and experimental diets by keeping the proportions of protein, fat and carbohydrate as nearly constant as possible. It was not possible to equalize roughage exactly. Milk was chosen as a standard high-value protein, and in alternate periods comparison was made between the nitrogen balances on cereal protein, supplemented with a small amount of milk (cream) and fruit proteins, and on milk protein supplemented in the same manner. The choice of these particular supplements was dictated by the importance of measuring the value of the cereal (breakfast food) protein under the usual conditions of eating these foods in American families.

The choice of milk as the source of the standard protein also would bring out values of importance in the transition from an all milk to a milk and cereal diet in the nutrition of infants.

Such a procedure, as will be seen, makes it possible to express the cereal protein in terms of milk protein—called here 'milk-replacement value.' This study is quite comparable in some respects to the studies of Sherman and Winters ('18, '19) on cornmeal and oat proteins. The method has proved its usefulness in other studies to be reported soon and therefore deserves publication even at this late date.

PREVIOUS WORK ON CEREAL PROTEINS

The first trustworthy reports on the quantity of dry substances 'utilized' (i.e., apparently digested and absorbed) from cereal products by the human alimentary tract, are those of Gustav Meyer (1871) and Rubner (1879) from the laboratory of Carl Voit. Both investigators conducted experiments on human subjects who ingested different sorts of white (wheat) and rye breads, with nothing else than butter and beer, for periods of 4 days. The 'utilization' figures are of little interest today because of changed dietary habits, improved methods of baking and more reliable chemical determinations. Since this early work there have been many investigations on human subjects regarding the apparent digestibility of breads and their nutritional value, the most important of which since 1900 doubtless are those of Woods and Merrill ('00), Newman, Robinson et al. ('12), Snyder ('00, '03), Hindhede ('14), Rubner ('16), the Food (War) Committee of the Royal Society of London ('18), Holmes ('19), Rubner ('18), Sherman ('20), French and Mattill ('35). A good summary of the studies of Rubner and his colleagues, occasioned by the World War, is given by Lusk ('28).

The principal interest in most of these studies was the relative digestibility of different breads, made from flours containing different proportions of the whole grain. In considering the utilization of the proteins practically no attention was given to the alimentary nitrogen, under the conviction,

of course, that it matters little what may be the source of the fecal nitrogen, whether the food or the alimentary organs, since it must be reckoned as loss in either case. In the assessment of 'biological values' of the proteins, as the term is used by Thomas and by Mitchell, the fecal loss must be correctly apportioned to food and alimentary products in order that the amount absorbed may be determined. If the necessary data are obtained to make this differentiation, one can readily calculate 'true' digestibility.

The necessary conclusion from all of the work on bread is that the coarser sorts (whole wheat, Graham, etc.) are considerably less digestible than the breads made from white flour. However, it should not be overlooked that practically no control diet work, in the sense of Mendel and Fine ('12) for example, has been done with breads. Apparent rather than true digestibility has been compared in all of these studies. Further, as strongly emphasized in the Report by the Food Committee of the Royal Society ('18), it does not follow that because bread of high extraction is somewhat less digestible than bread of low extraction, it is therefore less economical, either physiologically or financially.

The cereal breakfast foods offer certain advantages over breads for the determination of the value of cereal protein. They are available in pure form in hermetically sealed packages. Some are whole grain, some contain only the endosperm; some have all the bran removed, some contain a portion only of the bran; some are whole grain with germ of the grain added, but none, so far, have other than grain protein added. The effects of manufacturing processes can be studied.

The apparent digestibility of the cereal breakfast foods has been studied since 1900 by Woods and Snyder ('06), by Harcourt ('07) and Sherman and Winters ('18, '19). In the majority of the tests made by the first named, the cereals were eaten with cream and 'a little sugar,' and the digestibility of the cereal constituents was computed by the usual method of making allowance for the indigestibility of the

organic constituents of the cream, sugar being regarded as 100% digestible. In the case of the simple diet (just described) the range in the digestibility of protein was from 82.3% with granulated cornmeal to 91.6% with rolled oats. The lowest value for the digestibility of protein of the breakfast foods alone was 57.7% and was obtained from a specially prepared whole wheat product; the highest value, 85% with rolled oats. With the cereals alone, the lowest available energy value was 84.1% with a specially prepared 'whole wheat' cereal, and the highest was 94.4% with hominy.

Harcourt states that 'wheat germ' was more fully digested and absorbed than any other of the cereal foods. In this case, however, 'germ' is not used in the botanical sense. Probably the product was similar to the wheat endosperm employed in the present study. The protein of the cereals was less completely absorbed than other constituents, particularly with the high-priced, prepared foods like 'Force,' 'Orange Meat' and 'Norka.' "Certainly there is nothing in these results," says Harcourt, "to indicate that the high-priced, prepared breakfast foods are any more completely digested and absorbed than the wheat farinas and rolled oats or even than the old forms of granulated oatmeals."

These two studies were of great assistance in driving out of the market many highly sophisticated breakfast foods.

Sherman and Winters studied nitrogen balances with cornmeal and oatmeal as the principal source of nitrogen supplemented with milk and apple.

EXPERIMENTAL

This study embraced only three of the breakfast cereals most commonly used at the time, a wheat-endosperm product,[5] a pre-cooked rolled oats [6] and a 'whole wheat.' [7] In the control periods, whole cow's milk furnished 80% of the nitrogen, fruits 10% and XX cream 10% (table 1). Two fruits, prunes

[5] Cream of Wheat.
[6] 'Quick' Quaker Oats.
[7] Ralston's Wheat Cereal.

TABLE 1

Sample daily dietaries

SUBJECT	PERIOD	NUMBER OF DAYS	AVERAGE BODY WEIGHT	CEREAL	WHOLE MILK	CANE SUGAR	STR. BUTTER OIL	XX CREAM	CORN-STARCH	FRUIT		CALC. TOTAL CAL.	NITROGEN			
										Prunes	Bananas		Cereal or milk	Fruit	Cream	Total
			kg.	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*
J.C.	1st milk	3	70.3		840	262		386		66	337	2900	4.32	0.54	0.54	5.40
J.C.	Wheat endosperm	4	69.5	251		142	18	386		66	337	2900	4.32	0.54	0.54	5.40
R.A.	3rd milk	3	79.8		980	280		435		73	354	3180	4.90	0.58	0.61	6.09
R.A.	'Whole wheat'	4	80.0	253		213	21	435			436	3180	4.90	0.61	0.61	6.12
G.S.	2nd milk	3	66.0		690	284		335	40	100	260	2680	3.76	0.47	0.47	4.70
G.S.	Precooked oats	4	66.2	151		212	13	335		38	389	2680	3.76	0.47	0.47	4.70
J.M.	3rd milk	3	92.0		980	175		435	93	85	370	3178	4.90	0.61	0.61	6.12
J.M.	'Whole wheat'	4	91.6	253		167	15	435		85	370	3180	4.90	0.61	0.61	6.12

and bananas, were chosen because of their availability at all times and because by careful selection the constancy of the nitrogen could be assured. Frequent analyses were made not only of the fruits but of all constituents of the diet. After 3 days on the milk diet the milk protein was replaced with cereal protein and the cereal continued for 4 days. Then a second control period of 3 days, a second cereal period of 4 days, and so on. The fruit and cream continued the same throughout the control and cereal periods. Cornstarch was used in the control diets partly to reduce the large amount of sugar otherwise necessary in order to provide sufficient calories, and partly to give a little more bulk to the control diet in imitation of the cereal diet. To relieve monotony of flavor in the cereal periods, the cereal was served at lunch as a fried 'mush,' filtered butter oil being used as the frying fat. The cereal was cooked the day before and was placed in the ice box to cool. Having 'set' well, it was cut into slices and weighed before frying. Control periods showed no difference in utilization or N-balance occasioned by this change.

In this way the basal diet could be so constructed that the same articles, with the exception of the main source of protein, could be eaten each day, with only such minor adjustments of amounts, occasioned by the substitution of cereal proteins for milk proteins, and additions of cornstarch and butter oil, as were necessary to maintain the same intake of total protein, fat, carbohydrate and total calories throughout. The whole thought after the previous unpleasant experience was to find a method of assay on human subjects without resort to the unappetizing non-protein diet. In dealing with human subjects whether sick or well, it is unscientific to disregard the psychological effects of food. Colonic pains, except perhaps in author-subjects, can quickly ruin a built-up morale.

The stools of the several periods were separated by giving with the first meal of each period gelatin capsules containing charcoal or carmine. As a rule the two staining substances alternated with each other. The individual stools were well mixed in the collecting pails just after weighing and were sampled by difference of weight from weighing bottles.

There were no diarrheal stools, and occasional loose stools gave no evidence of excessive fermentation.

RESULTS

In table 2 are given the data necessary for calculating apparent and true digestibility of the cereal proteins consumed. The latter differs from the former only in the use made of the alimentary fecal nitrogen. It is included as part of the waste from the cereal nitrogen (apparent) or it is subtracted from total fecal nitrogen to obtain the 'true' food waste. By analogy with in vitro experiments where deduction must be made in a blank for the nitrogen of the enzyme preparation used, so in the living subject something must be taken off for the nitrogen contributed to the feces by the digestive secretions in order to get the true digestibility of the food.

The values for true digestibility in this study are only approximately correct, because the alimentary or 'endogenous' fecal nitrogens were not determined in the same study. Thus, subjects J.M., J.C. and R.A. were also subjects in the earlier study and had changed but little in weight. Since the basal diet used in that study was substantially the same as that used with the cereal in the replacement value study and was eaten at the same time of year (July and August) it may be assumed that the average alimentary nitrogen of the former study (exclusive of the watery stools) would have been very nearly the same in the latter. The figures for alimentary nitrogen, therefore, are borrowed from that study. G.S. was a subject somewhat smaller than J.C. and ate the same number of calories per kilogram of body weight; his alimentary nitrogen, therefore, is estimated from that of J.C. by proportion of his calories to those of this subject. The contribution of the fruit and cream to the fecal nitrogen in this study is estimated from the coefficients of digestibility for these foods given by Atwater ('04). The apparent digestibility of the cereal proteins is calculated by finding the percentage which the cereal N of the feces represents of the cereal N consumed. In several instances there was no food

TABLE 2

Apparent and true digestibility of cereal proteins

CEREAL	SUBJECT	FOOD NITROGEN			FECES NITROGEN			PER CENT APPARENT DIGESTIBILITY OF CEREAL N	ALIMENTARY [2] N	NET CEREAL N IN FECES	PER CENT 'TRUE' DIGESTIBILITY OF CEREAL N
		Total	Fruit and cream	Cereal	Total	Fruit and cream [1] (estd.)	Other N				
Wheat endosperm	J.M.	6.12	1.22	4.90	0.98	0.11	0.87	82	1.40	0.0	100
	J.C.	5.40	1.08	4.32	1.19[3]	0.09	1.10	74	1.00	0.1	98
	R.A.	6.12	1.22	4.90	0.91[3]	0.11	0.80	84	1.24	0.0	100
	G.S.	4.70	0.94	3.76	1.05	0.08	0.97	74	0.92	0.05	99
							Average	78		Average	99
Precooked oats	J.M.	6.12	1.22	4.90	2.21	0.11	2.10	57	1.40	0.70	85
	J.C.	5.40	1.08	4.32	2.25	0.09	2.16	50	1.00	1.07	75
	R.A.	6.12	1.22	4.90	1.30	0.11	1.19	76	1.24	0.0	100
	G.S.	4.70	0.94	3.76	1.69	0.08	1.61	57	0.92	0.69	82
							Average	60		Average	85
'Whole wheat'	J.M.	6.12	1.22	4.90	2.23	0.11	2.12	56	1.40	0.72	85
	J.C.	5.40	1.08	4.32	1.63	0.09	1.54	64	1.00	0.54	87
	R.A.	6.12	1.22	4.90	1.73	0.11	1.62	67	1.24	0.38	90
	G.S.	4.70	0.94	3.76	1.66	0.08	1.58	58	0.92	0.66	82
							Average	61		Average	86

[1] Apparent digestibility of fruit N = 85%; of cream 97% (Atwater).

[2] Alimentary N for subjects J.M., J.C. and R.A., average from unreported study; for subject G.S. estimated by weight and proportion of calories.

[3] Average of two periods.

There were no diarrheal stools, and occasional loose stools gave no evidence of excessive fermentation.

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		Total	Fruit and cream	Cereal	Total	Fruit and cream [1] (estd.)	Other N				
Wheat endosperm	J.M.	6.12	1.22	4.90	0.98	0.11	0.87	82	1.40	0.0	100
	J.C.	5.40	1.08	4.32	1.19[3]	0.09	1.10	74	1.00	0.1	98
	R.A.	6.12	1.22	4.90	0.91[3]	0.11	0.80	84	1.24	0.0	100
	G.S.	4.70	0.94	3.76	1.05	0.08	0.97	74	0.92	0.05	99
							Average	78		Average	99
Precooked oats	J.M.	6.12	1.22	4.90	2.21	0.11	2.10	57	1.40	0.70	85
	J.C.	5.40	1.08	4.32	2.25	0.09	2.16	50	1.00	1.07	75
	R.A.	6.12	1.22	4.90	1.30	0.11	1.19	76	1.24	0.0	100
	G.S.	4.70	0.94	3.76	1.69	0.08	1.61	57	0.92	0.69	82
							Average	60		Average	85
'Whole wheat'	J.M.	6.12	1.22	4.90	2.23	0.11	2.12	56	1.40	0.72	85
	J.C.	5.40	1.08	4.32	1.63	0.09	1.54	64	1.00	0.54	87
	R.A.	6.12	1.22	4.90	1.73	0.11	1.62	67	1.24	0.38	90
	G.S.	4.70	0.94	3.76	1.66	0.08	1.58	58	0.92	0.66	82
							Average	61		Average	86

[1] Apparent digestibility of fruit N = 85%; of cream 97% (Atwater).

[2] Alimentary N for subjects J.M., J.C. and R.A., average from unreported study; for subject G.S. estimated by weight and proportion of calories.

[3] Average of two periods.

nitrogen, as estimated, and therefore the true digestibility is 100%.

The values for apparent digestibility are disappointingly low compared with those given by Woods and Snyder and by Harcourt as 'coefficients of digestibility' for similar foods. For example, Woods and Snyder give for 'oat products' an average coefficient for the protein content of 77.9%. In table 2 for four subjects on precooked oats the value is 60%. Woods and Snyder give also for 'wheat products, uncooked, decorticated' a value for protein of 80.9%. The value in table 2 for wheat endosperm, presumably a comparable product except that it was cooked, is 78%. Harcourt gives for rolled oats and wheat farinas 80 and 72.5%, respectively.

The main reason for the lower values in the work reported here is the lower level at which the protein was fed. The most favorable level for exhibition of the nutritive value of the protein (which was the determining reason for the low level) plainly is not the most favorable for exhibition of a high coefficient of digestibility. Another reason for the difference may be the rather liberal amounts of fruit eaten throughout this study. The correction for alimentary nitrogen from Atwater's work may not do full justice to the cereal products used, in view of the lower level of intake in the present work.

Comparing the cereals among themselves, it is found that wheat endosperm leads the list both for apparent and true digestibility of protein. It is followed by 'whole wheat' and precooked oats, the two differing but slightly. The order is the same for apparent and true digestibility. This fact seems to confer additional validity on the method of comparison.

The results of this study confirm those obtained in all the principal investigations on breads of different kinds quoted above. The proteins of the cereal product with the least roughage are the most digestible, although the differences often are so small as to be negligible. The same order of digestibility of the proteins was obtained in the studies of

artificial digestion (paper II of this series, Carman, Smith et al., '29); the effect of coarseness upon digestibility in the rat is discussed in paper III (Mattill and Smith, '30).

From the data in tables 2 and 3, the apparent and true digestibility of the milk proteins in the four subjects were

TABLE 3

Average daily nitrogen balances—all periods in order of their occurrence

SUBJECT	PERIOD	NUMBER OF DAYS	N EXCRETION			TOTAL FOOD N	N BALANCE
			Urine	Feces	Total		
J.M.	1st milk	3	6.31	2.33	8.64	6.12	— 2.52
	Wheat endosperm	4	5.89	0.98	6.87	6.12	— 0.75
	2nd milk	3	5.25	2.12	7.37	6.12	— 1.25
	Precooked oats	4	5.71	2.21	7.92	6.12	— 1.80
	3rd milk	3	4.68	1.92	6.60	6.12	— 0.48
	'Whole wheat'	4	5.01	2.23	7.24	6.12	— 1.12
	4th milk	3	4.61	1.90	6.51	6.12	— 0.49
J.C.	1st milk	3	6.35	1.76	8.11	5.40	— 2.71
	Wheat endosperm	4	6.49	1.41	7.90	5.40	— 2.50
	2nd milk	3	4.38	1.13	5.51	5.40	— 0.11
	Precooked oats	4	4.96	2.25	7.21	5.40	— 1.81
	3rd milk	3	4.29	1.34	5.63	5.40	— 0.23
	'Whole wheat'	4	5.15	1.63	6.78	5.40	— 1.38
	4th milk	3	4.79	1.45	6.24	5.40	— 0.84
	Wheat endosperm	4	5.64	0.97	6.61	5.40	— 1.21
R.A.	1st milk	3	6.51	2.35	8.86	6.12	— 2.74
	Wheat endosperm	4	6.67	1.10	7.77	6.12	— 1.65
	2nd milk	3	4.31	1.71	6.02	6.12	+ 0.10
	Precooked oats	4	4.90	1.30	6.20	6.12	— 0.08
	3rd milk	3	4.83	1.83	6.66	6.12	— 0.54
	'Whole wheat'	4	6.05	1.73	7.78	6.12	— 1.66
	4th milk	3	4.35	1.54	5.89	6.12	+ 0.23
	Wheat endosperm	4	5.10	0.72	5.82	6.12	+ 0.30
G.S.	1st milk	3	6.28	1.63	7.91	4.70	— 3.21
	Wheat endosperm	4	6.16	1.05	7.21	4.70	— 2.51
	2nd milk	3	5.09	1.59	6.68	4.70	— 1.98
	Precooked oats	4	4.86	1.69	6.55	4.70	— 1.85
	3rd milk	3	5.27	1.13	6.40	4.70	— 1.70
	'Whole wheat'	4	5.25	1.66	6.91	4.70	— 2.21
	4th milk	3	5.32	1.83	7.15	4.70	— 2.45

calculated in the same way as for the cereal proteins. In the order of the subjects the figures for apparent digestibility are 60, 60, 64 and 62, average 64; for true digestibility they are 80, 92, 89 and 88, average 87. It is evident that the cereal proteins compare very favorably, so far as digestibility alone is concerned, with those of whole cow's milk.

Some element of supplementation as regards digestibility as well as ultimate nutrient efficiency of the proteins probably is concerned here. This is a subject which has not been studied sufficiently to form an adequate basis of comment. Woods and Merrill ('00) found that bread gave a higher coefficient of digestibility when eaten with milk than when eaten alone. Quite possibly the cream, starch, sugar and fruit eaten in this study supplemented cereal proteins as regards digestibility better than they do milk protein. The unfavorable effect of starch on the digestibility of protein observed by Schulze and Märcker (1871) and by Mendel and Lewis ('13) also may have been a factor in the poor showing made by milk proteins in the control periods, for subject J.M. who gave the lowest average values both for apparent and true digestibility, ate the largest amount of cornstarch (table 1), subject G.S. who ate the next largest amount, gave second lowest, and subject J.C. who ate none, gave the highest values. The cornstarch was taken for the most part as a blanc mange pudding, occasionally raw (Langworthy and Deuel, '22).

Since the whole wheat and oats products show very nearly the same true digestibility, a fairly good basis for comparing the biological values of wheat and oats proteins is provided.

NITROGEN BALANCES

The separation and handling of the feces has been described on page 23. Urines were collected in 24-hour periods and analyzed for total nitrogen. The nitrogen balances are presented in table 3. The order of the three cereals alternating with milk is the same for all subjects; but for the purpose of testing the influence of position in the series on nitrogen balance, with two subjects (J.C. and R.A.) the wheat

endosperm was repeated at the end of the series. It is evident that the position in a series of low protein diets does influence the nitrogen balance. This appears not only in comparing the two wheat endosperm periods but also in comparing the several milk periods among themselves. Twice as often as not, the later the milk period occurs in the series, the more favorable is the N-balance. This result was not unexpected because of the similar experience of one of us (Murlin, '07) in a study of the nutritive value of gelatin. The greater negative balances at the beginning doubtless indicate that the adjustment period on milk before collections of excreta began (2 days) was not long enough. And the same circumstance probably influenced slightly at least the first wheat endosperm period. The principal reason, however, is the continuously increasing need of protein to make good the growing deficit.

Of the fourteen periods with cereal protein ten exhibit a greater negative balance than the average of adjacent milk periods: three out of six with wheat endosperm, three out of four with precooked oats, and all four with 'whole wheat.' Fecal nitrogen was higher with precooked oats and 'whole wheat' than in the adjacent milk period; it was less in all wheat endosperm periods than in adjacent milk periods. In eleven out of fourteen periods the urinary N was higher in the cereal periods than the average of the adjacent milk periods supplying the same N intake.

The absolutely even level of N intake throughout such a study would seem to furnish a more normal set of conditions for a test of the influence of food proteins on metabolism than could exist with the sudden shifts to low N control periods as in the experiments of Thomas.

REPLACEMENT VALUE

Reasons have already been given (p. 18) for adopting the method which gives this value. The name, abbreviated R.V., is given to the value of the cereal protein as compared with milk as standard protein. Table 4 contains in the third

TABLE 4

Replacement value vs. biological value of cereal proteins. Second study

SUBJECT	DIFFERENCE BETWEEN MILK AND CEREAL PERIODS		CEREAL N IN FOOD	REPLACEMENT VALUE	ALIMENTARY N	CEREAL N			BIOLOGICAL VALUE
	Urine N [1]	N balance [2]				In feces	Absorbed	In urine	
				Wheat endosperm					
	gm.	*gm.*	*gm.*	%		*gm.*	*gm.*	*gm.*	%
J.M.	+ 0.11	+ 1.13	4.90	100	1.40	0.0	4.90	0.11	98
J.C.	+ 1.13	— 1.09	4.32	75	1.00	0.1	4.22	1.13	73
R.A.	+ 1.26	— 0.33	4.90	95	1.24	0.0	4.90	1.26	74
G.S.	+ 0.48	+ 0.08	3.76	100	0.92	0.05	3.71	0.48	87
J.C.	+ 0.85	— 0.37	4.32	86	1.00	0.0	4.32	0.85	80
R.A.	+ 0.75	+ 0.07	4.90	100	1.24	0.0	4.90	0.75	85
				Av. 93					Av. 83
				Precooked rolled oats					
J.M.	+ 0.75	— 0.26	4.90	95	1.40	0.70	4.20	0.75	82
J.C.	+ 0.63	— 1.64	4.32	62	1.00	1.07	3.25	0.63	80
R.A.	+ 0.38	+ 0.14	4.90	100	1.24	0.0	4.90	0.33	93
G.S.	— 0.32	— 0.01	3.37	100	0.92	0.69	2.68	0.0	100
				Av. 89					Av. 89
				'Whole wheat'					
J.M.	+ 0.37	— 0.64	4.90	87	1.40	0.72	4.18	0.37	91
J.C.	+ 0.61	— 0.85	4.32	80	1.00	0.54	3.78	0.61	84
R.A.	+ 1.46	— 1.50	4.90	69	1.24	0.38	4.52	1.46	68
G.S.	— 0.04	— 0.14	3.76	96	0.92	0.66	3.10	0.0	100
				Av. 83					Av. 85

[1] +, signifies increase of urine N due to cereal; —, a decrease.

[2] +, signifies improvement in N balance; —, indicates adverse effect.

column the average differences in N balance obtained with the cereal protein as compared with milk. When this difference is positive (+) it indicates better total utilization than with milk. Better absorption would leave less fecal nitrogen; or greater retention after absorption would mean less urinary nitrogen and more replacement of the wear and tear protein of the body. Whichever the cause of an improved N balance, the cereal protein is given a R.V. rating, in comparison with milk, of 100%. When the difference in column 3 is minus, the R.V. of the cereal is calculated by finding the percentage of the cereal nitrogen fed which the figure represents and subtracting from 100. Thus in the second line for subject J.C. the difference is —1.09. This is 25% of the cereal N fed. The replacement value is therefore 75%.

For comparison the biological value (B.V.) is calculated by Mitchell's method, i.e., by subtracting the alimentary nitrogen from the 'other N' of table 2, to find the fecal N due to the cereal. This cereal residue N subtracted from the cereal N fed gives the amount absorbed. The B.V. (table 4) then is found by calculating the percentage of this absorbed N represented by the cereal N in the urine and subtracting from 100.

Wheat endosperm had the highest replacement value, precooked oats lower and 'whole wheat' lowest. The high rank of the first is explained largely by the low fecal nitrogens which it produced (table 3). For each subject it was lowest of the three cereals in this respect. On the other hand, with one exception ('whole wheat' for subject R.A.), the urinary nitrogen was higher from this cereal than from the others.

The replacement value of wheat endosperm for each of the two subjects who ate it in a second period (J.C. and R.A.) was greater in the second period because of lower fecal as well as lower urinary N excretion. Longer sustenance on low protein accounts for the better utilization in both respects. Curiously enough the repetition of this cereal did not affect its average replacement value (93); the average of the four first trials is 92.5; the B.V. also was not changed by the repetition. For the oats product the replacement value and

the biological value are the same, and for 'whole wheat' nearly the same.

In the earlier unreported study, the biological values of corn endosperm (cornflakes), wheat endosperm and regular (not precooked) rolled oats were 84, 73 and 97, respectively. On account of diarrheal stools, several periods had to be excluded and the separation of feces in some of the remaining periods was none too satisfactory. The value for rolled oats is altogether out of line with that found for the precooked product, in this study (89), and that for wheat endosperm is much lower than the figure found in the present study. In view of the contained roughage the value for precooked oats in table 4 seems too high in comparison with whole wheat. The differences between these two studies cannot be accounted for by the different levels at which the proteins were eaten, for they were nearly the same. With rats, Mattill ('30) found biological values of 73 for the wheat endosperm, 72 for the 'whole wheat' and 82 for the precooked oats used in this study. Both sets of values are somewhat higher than most of those previously reported in the literature (see table by Boas-Fixsen, '35) but those of the present study are consistent with the egg-replacement values to be reported in the following paper (Sumner, Pierce and Murlin, '38).

SUMMARY AND CONCLUSIONS

1. The apparent and 'true' digestibility in the human alimentary tract of the proteins of three cereal breakfast foods have been studied, namely, a wheat endosperm product, a so-called 'whole wheat,' and a precooked rolled oats.

2. The nutritive value of the proteins was studied by a milk replacement method. This consisted of feeding the cereals in alternate periods with milk, providing 80% of the nitrogen, while 10% in both diets was supplied by cream and 10% by fruit.

3. Alimentary or 'metabolic' nitrogen of the feces was determined in an unreported study and was found to be roughly proportional to body weight, total calories being also in that proportion.

4. Apparent and 'true' digestibility of the different cereals parallelled each other, and for the most part the digestibility, whether apparent or true, was in the same order for the different subjects.

5. The results confirm those of previous investigators in finding the apparent digestibility of endosperm products distinctly higher than that of whole grain products. 'True' digestibility, which undertakes to apportion fecal nitrogen correctly to food residues and alimentary products, gives obviously a higher ranking to all, but does not change notably the relative ranking of the products used.

6. The milk-replacement value of the proteins of the precooked rolled oats and the 'whole wheat' were substantially the same numerically as their biological values. That of wheat endosperm was somewhat higher than its biological value.

7. The replacement value method offers distinct advantages for experiments on human subjects because at all times the diet is tolerable and gives separable stools.

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THE EGG-REPLACEMENT VALUE OF SEVERAL PROTEINS IN HUMAN NUTRITION

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Because of the unpalatability of the nitrogen free diet and its tendency to induce diarrhoea in human subjects, Murlin and Mattill ('26, '38) have suggested that some well-utilized protein such as milk be included in a standard diet consisting largely of cornstarch, sugar, cream and fruit and a comparison made of the ability of other protein foods to replace the milk nitrogen. When such a diet furnished between 5 and 6 gm. of nitrogen, 80% of which came from the milk, and the remaining 20% equally divided between heavy cream and fruit, these investigators found that a whole oats product replaced milk protein slightly better than a 'whole wheat,' but both were exceeded by wheat endosperm.

French and Mattill ('35) using this method and feeding at a 6 gm. level of nitrogen intake, report that the nitrogen of white, whole wheat or rye bread is able to replace milk nitrogen to the extent of 83%.

The investigation of Rose and MacLeod ('25) is very similar in technic and their results for meat and for a combination of bread and milk can be expressed in terms of replacement value using milk as a standard. The average of the meat periods for three subjects shows a replacement value of 93; for the one subject on bread and milk an average of 96.

[1] The data in this paper are taken from a thesis submitted in partial fulfillment of the requirements for the doctorate of philosophy.

PLAN OF INVESTIGATION

In the present investigation, egg protein was chosen as the standard to be replaced by the other foods studied. This choice was influenced by the fact that of all of the foods studied by Mitchell, egg gave the highest biological value in the nutrition of young rats. The ability of powdered whole milk, fresh pasteurized milk, the combination of milk and fresh baker's yeast, egg and yeast and a wheat endosperm [2] product were tested as to their ability to replace the nitrogen of fresh whole egg in the nutrition of human subjects.

Ten healthy adults, two women and eight men, served as subjects. The group continued their routine duties throughout the experiment, any additional exercise being kept as uniform as possible.

Body weights were obtained just prior to breakfast each day, and there was some loss recorded for each subject over the 40 days of the diet. Richet ('28) claims that loss in body weight occurs in human subjects fed a diet of sufficient calories but low in protein. During the experiment, the weather became decidedly warmer and several of the men, notably those who appeared to lose the most weight, changed into lighter clothing. If this change of clothing were taken into consideration, the greatest loss for any subject would not be more than 1 or 2 kg. which falls within the daily variation in body weight expected for that individual.

On the low nitrogen diet used by Sumner and Murlin ('38) in the determination of the biological value of milk and egg protein, the daily excretion of urinary and fecal nitrogen approximated 4 gm. for the men and 3 gm. for the women. These figures were chosen as the amounts of nitrogen to be supplied by the food during the present study. It was hoped that at this level of feeding, any difference between individual proteins might be more readily distinguished. In a replacement value study on two young men D and R, carried out simultaneously with the biological value determination just

[2] Cream of Wheat.

mentioned, with a nitrogen intake of 6.5 gm., milk nitrogen replaced egg nitrogen 100% [3] (table 4).

Numerous feeding experiments with animals have shown that at the higher planes of protein intake, a low percentage content of some essential amino acid may be masked, also that a difference in the ability to support growth may be noted between two proteins fed at a level near the minimum requirement, but not at a higher level. For this reason the lower level was chosen for the present study.

As that part of the food intake which consisted of cornstarch, sucrose, lactose with small additions of fruit, was the same for each subject throughout the experiment, it is referred to as the basal diet. This basal diet plus egg is designated as the standard diet. The basal diet supplied from 0.4 to 0.6 gm. of nitrogen, varying with different individuals, but kept constant for the same subject throughout the experiment. The nitrogen in excess of that supplied by the basal diet came from egg or from the protein under consideration, and amounted to 82% of the total nitrogen of the standard diet for the women, and 86% for the men. Such articles of the diet as cornstarch, lactose, vitamin B concentrate tablets (Harris), wheat endosperm (two lots) gum acacia, hydrogenated cottonseed oil,[4] salad oil, corn syrup,[5] canned peaches and tomato juice were ordered in sufficient amounts to last through the experiment. Through the cooperation of a local dairy, milk and cream were obtained from the same herd. Before measuring out the quantity due each subject, all the milk, as well as all the cream, was separately pooled and well mixed. Samples were taken for analysis at frequent intervals. A quantity of butter sufficient for the duration of the diet was especially made by the dairy from one batch of cream. The butter was stored in a refrigerator until used. Fresh eggs were brought in every day from a farm, where the hens received a diet of constant composition. The eggs

[3] More extensive investigations at this higher level will be reported from this laboratory at a later date.

[4] Crisco.

[5] Karo.

required for each meal were broken into a large dish and thoroughly mixed, before the individual amounts were weighed out for the various subjects. The egg was cooked and served as a plain omelet. Frequent determinations for nitrogen were made on the raw mix. Eggs, milk and cream showed a remarkably uniform total nitrogen content.

The daily distribution of food into meals was as follows:

Breakfast

Periods 1 to 5 inclusive	*Period 6*
Grapefruit with sucrose	Grapefruit with sucrose
Coffee	Coffee
Cornstarch bread * with butter and corn syrup	Cream of Wheat cereal with cream and sucrose
Lactose lemonade	Lactose lemonade

Lunch

Egg omelet or milk or milk and yeast or egg and yeast	Fried cream of wheat with butter and corn syrup
Cornstarch bread with butter and corn syrup	Lactose lemonade
Lactose lemonade	Canned peaches
Canned peaches and cream	

Supper

Egg omelet or milk or milk and yeast or egg and yeast	Cream of Wheat cereal with cream and sucrose
Cornstarch bread with butter and corn syrup	Head lettuce with oil and lemon juice
Lactose lemonade	Lactose lemonade
Head lettuce with oil and lemon juice	Corn syrup

The total amounts ingested are given in table 1 for one subject, selected as being typical of the group. Due to the omission of the cornstarch bread during the wheat endosperm period, the proportions of certain food stuffs differ in period 6 from those in period 1. Four subjects substituted tomato juice for grapefruit. Adjustment of calories was made by

* For composition of this bread and mode of preparation see paper by Sumner. and Murlin ('38).

adding to, or subtracting from, the total amount of sucrose in the basal diet. The vitamin B tablets were omitted during all the yeast periods.

TABLE 1

Daily food intake—subject J

	PERIODS 1 AND 4 EGG	PERIOD 2 [1] MILK	PERIOD 3 [1] MILK AND YEAST	PERIOD 5 [1] EGG AND YEAST	PERIOD 6 WHEAT ENDOSPERM
Graptfruit	gm.	gm.	gm.	gm.	gm.
Pulp	80				120
Juice	20				60
Coffee	2/3 c.				2/3 c.
Canned peach					
Pulp	80				120
Juice	20				40
4X cream	14				169
Lettuce	100				100
Salad oil	30				30
Lemon juice	45				45
Lactose	36				36
Sucrose	151	113	113		176
Cornstarch	150				None
Crisco	20				None
Sucrose	15				None
Karo	17				None
Butter	40				25
B conc. tablet	1		0	0	2
Karo	140				120
Egg	180	None	None	None	None
Milk, dry	None	85	66	None	None
Egg	None	None	None	141	None
Yeast	None	None	3 cakes	3 cakes	None
Wheat endosperm	None	None	None	None	160
Total N	4.25	4.09	4.04	A 4.19 B 4.29	A 3.81 B 3.86
Total Cal.	3140	3164	3113	3131	3129

[1] Amounts of food eaten during periods 2 to 5 inclusive are the same as for period 1 unless otherwise noted.

Coffee was allowed once a day if desired. In order to insure a uniform beverage, a brand of powdered coffee [7] was employed. For the six subjects who took coffee, the same amount of powder was always weighed out on a chemical balance, and

[7] George Washington.

the same measure of boiling water added. In the yeast periods one and one-half cakes of fresh baker's yeast suspended in water were taken before the noon and evening meals, usually ½ hour before eating. Water intake was kept as constant as possible. All servings of food were weighed to the nearest gram on standard spring scales, designed for dietetic work and graduated in gram divisions.

The experiment was divided into five parts:

Period 1. Egg as the source of protein. For 3 days preceding this period, the food of the two women subjects consisted mainly of potatoes. After 1 day on the standard diet, the daily excretion of urinary nitrogen was followed. The actual collection period for urine and feces was 8 days. All the men subjects went from their customary mixed diet directly onto the standard ration. After 1 preliminary day, daily determinations were made for urinary nitrogen. The collection period for the urine and feces lasted 7 days.

Period 2. Milk, either pasteurized or as whole milk powder,[8] served as the source of protein, the amount of nitrogen being equivalent to that received from the egg during the standard diet. A fore-period of 3 days preceded the 5-day collection period for urine and feces.

Period 3. Milk and yeast. Approximately 0.8 gm. of the milk nitrogen was replaced by an equivalent amount of nitrogen from fresh bakers' yeast. Collections were made over a 4-day period. The original plan had been to continue the milk and yeast period for another 4 days, but due to the complaints concerning the unsatisfying and almost nauseating qualities of both the milk, and the milk and yeast diets, it was thought unwise to persist longer. This difficulty in the milk periods may have been psychological. The odor and taste of the hot egg omelet stimulated an interest in food and gave a feeling of well-being, which was never felt when milk supplemented the basal diet. It is interesting to note that Martin and Robinson also complained of the difficulty of ingesting the basal diet during their experiments on milk.

Period 4. Second egg period. Urine and feces were collected for a 4-day period.

Period 5. Egg and yeast protein. Eight-tenths of a gram of the egg nitrogen was replaced by an equal amount of nitrogen from fresh bakers' yeast. The experiment lasted 6 days,

[8] Klim.

consisting of two periods of 3 days each. Urine and feces were collected for each period.

Period 6. Wheat endosperm[9] as the source of protein. The cornstarch bread was dropped from the diet with the introduction of the cereal. The experiment was divided into 2 parts, each consisting of 5 days, separate collections being made for each period.

Urines were collected over 24-hour periods, from 8.00 A.M. to 8.00 A.M. The urinary nitrogen was averaged for the last 2 days of each period, and this figure used in the calculations.

Charcoal was used for separating the feces of the different periods. Unless otherwise noted, the stools were normal. All stools were collected directly into ½-gallon enamel buckets, by means of special commodes. The covered pails were kept in the cold room until the contents could be weighed, thoroughly mixed and sampled. This took place as soon as possible after the stool was passed. One-tenth by weight of every stool passed during each diet period was pooled for nitrogen determination. These samples were placed in a 3-liter Florence flask with concentrated H_2SO_4. After digesting to a fairly homogeneous mixture, the solution was transferred quantitatively to a volumetric flask, cooled in the cold room and made up to volume at the same temperature. Total nitrogen was determined in the fecal digest and in the daily urine samples by the macro-Kjeldahl method, all analyses being run in duplicate.

The weights of samples for the determination of moisture were obtained by difference from portions of the well-mixed feces, which had been placed in glass-stoppered weighing bottles. These samples of approximately 3 gm. were placed in small evaporating dishes covered with ethyl alcohol and evaporated to dryness on the steam bath. The dishes and their contents were then placed in an electric oven at 100° and brought to constant weight.

[9] See footnote 2, page 38.

EXPERIMENTAL RESULTS

The averaged nitrogen balances for the group are presented for each protein period in table 2. The figures given for the daily nitrogen content of the ingested food and of the feces were obtained by subtracting the amount of nitrogen originating from the gum acacia. This deduction has been made on the assumption that gum acacia nitrogen is not digested. A correction for both dietary and urinary nitrogen has also been made for those subjects who took coffee during the experiment.

TABLE 2

Average N balances for replacement value study

NUMBER OF SUBJECTS	EGG I	MILK				MILK AND YEAST			
		Number of subjects	Dry	Number of subjects	Fresh	Number of subjects	Dry	Number of subjects	Fresh
	gm.		*gm.*		*gm.*		*gm.*		*gm.*
Women 2	−0.91	1	−0.34	1	−0.15	1	+0.15	0[1]	....
Men 7	−1.31	4	−1.04	3[2]	−1.09	4	−1.79	4	−1.14
Average	−1.23		−0.90		−0.86		−1.40		−1.14
	EGG II	EGG AND YEAST				WHEAT ENDOSPERM			
		A		B			A		B
Women 2	+0.06	2	−0.13	2	−0.17	1[2]	−0.54	2	+0.09
Men 8	−0.39	8	−0.86	8	−0.91	7[2]	−1.06	8	−0.96
Average	−0.30		−0.72		−0.77		−0.99		−0.86

[1] Nausea and catamenia.

[2] One subject excluded because of irregularity.

Egg as the source of protein. For each individual, as well as for the average of all ten subjects, the nitrogen balance, —0.30 gm., of the second egg period is more favorable than that of the first, —1.23 gm. This difference is due in all probability to the fact that the subjects had not yet reached their minimal nitrogen excretion at the end of the first period. Hence for purposes of comparison and for the calculation of replacement values, the figures of the second egg period will be used.

Milk as source of protein. During the milk period half of the group received fresh pasteurized milk and the other half a whole milk powder.[10] The average balance for the five subjects taking dry milk was —0.90 gm., for the four taking fresh milk —0.86, showing no difference in utilization of the two kinds of milk nitrogen. Comparing the utilization of milk nitrogen with that of egg, the individual milk balances are less favorable than those of the second egg period in every instance. Even the average of the two egg periods, —0.76 gm. is smaller than that for either of the two kinds of milk.

Milk and yeast as source of protein. Reports concerning the biological value of yeast protein have varied so much (Pierce, '32) that it seemed worth while to try the replacement method. In this period, one of the subjects of the fresh milk group became ill and had to discontinue the diet for a few days. If the average balance, —1.14 gm. for the four men on fresh milk and yeast be compared with the average for three of the four on fresh milk alone, —1.09 gm., the difference is negligible. Using the average for the four subjects on fresh milk alone, —0.86, there is an advantage of milk alone but the result is obviously due to the very low minus balance of the woman subject. For the five subjects on the reconstructed dry milk plus yeast, there is again a much less favorable average balance than for milk alone, the former being —1.40 gm. and the latter —0.90 gm. One subject on dry milk, and two subjects on fresh milk had more favorable nitrogen balances when yeast nitrogen replaced part of the milk nitrogen; for all the others the nitrogen balance of the milk period was superior.

Egg and yeast as source of protein. The average balance of either of the two egg-and-yeast periods, —0.72 and 0.77 gm., is less favorable than that of the second egg period, —0.30 gm. With one exception, the individual balances for the first part of the egg-and-yeast assay are all less favorable than for egg alone. This also holds for seven out of ten subjects during the second part of the experiment; for the

[10] See footnote 8, page 42.

other three subjects there is a slightly greater retention of nitrogen on egg and yeast. If the comparison had been made between the average balances of the two egg-and-yeast periods, —0.75 gm., and the average of both egg periods, —0.77 gm., the conclusion that there is no difference in nitrogen utilization would have seemed justified.

Wheat endosperm as source of protein. The average minus balances of both wheat endosperm periods, —0.99 and —0.86 gm., are definitely greater than that of the second or even than the average of the two egg periods. The second wheat endosperm balance is identical with that of fresh milk, —0.86 gm. It must be borne in mind that from 1.5 to 2.0% of the total nitrogen of this diet came from 4X cream, so there is in all probability a slight supplementary effect between the two sources of nitrogen. Sherman et al. ('18) and Sherman ('20) have noted the small amount of milk nitrogen necessary to induce a positive nitrogen balance with various cereal grains. In the second period of this study, two of the eight subjects had a nitrogen retention as good as that on egg. This is in line with the work of Osborne and Mendel ('19), who observed that the protein of white flour reinforced with wheat gluten was adequate for maintenance of adult rats.

Discussion

In the majority of subjects, egg protein was superior to any of the others in lowering the negative nitrogen balance. If the different periods for each individual are compared to the second egg period, the following variations may be noted: the milk and yeast period of one subject showed a retention of 0.09 gm. greater than for egg; four subjects attained slightly more favorable balances in the egg-and-yeast periods, the difference in favor of the latter being 0.05, 0.08, 0.25 and 0.04 gm. of nitrogen, respectively; for two subjects during the second wheat endosperm period, the nitrogen balance was on a par with that of egg.

Out of ten cases egg was superior to milk in every instance; to milk and yeast in eight out of nine cases; to wheat endosperm plus the small amount of cream in sixteen out of eighteen cases; to egg and yeast in sixteen out of twenty cases.

To express these results on a percentage basis, the replacement value of the different proteins has been calculated, according to Murlin and Mattill ('38) using the second egg period as a standard. The difference between the nitrogen balance of the standard, and of the experimental protein period is expressed as a percentage of the egg nitrogen fed in the former period. This figure subtracted from 100, represents the replacement value. Should the difference between the

TABLE 3

Summary of egg-replacement values

Protein	*Number of subjects*	*R.V.* Average, %	Range
Dry milk	5	85	76– 93
Fresh milk	4	82	65– 92
Dry milk and yeast	5	72	47–100
Fresh milk and yeast	4	76	69– 84
Egg and yeast I	10	88	73–100
Egg and yeast II	10	86	57–100
Wheat endosperm I	8	82	54– 98
Wheat endosperm II	8	87	47–100

balances be positive, the experimental protein is said to have a replacement value of 100%. For example, the computation for dry milk, for one subject is as follows:

Bal. 2nd egg period, gm.	*Bal. dry milk period, gm.*	*Difference, gm.*	*Egg N gm.*	*R.V., %*
+ 0.06	— 0.034	— 0.40	2.42	84

The average replacement values of the different proteins for all subjects are given in table 3.

Comparison on this basis shows but little difference in the ability of the two varieties of milk to replace egg nitrogen. The substitution of yeast for part of the milk protein lowers the replacement value below that of milk alone. The egg and yeast combination is not so good as egg. Wheat endosperm with the small amount of 4X cream is able to replace egg nitrogen equally as well as milk.

For comparison with the preceding results at a level of 3 to 4 gm. N intake daily the results on the two student subjects D and R are presented as set forth in detail in table 4. Egg and milk protein supplemented in the same manner were fed in successive 7-day periods at a level of approximately 6.5 gm. N daily. A final egg-protein period of 3 days terminated the experiment in the same manner for both subjects.

TABLE 4

Replacement value of milk in terms of egg protein at a 6.5 gm. N intake

	N INTAKE			N OUTPUT			N BAL.
	Basal	Protein	Total	Urine	Feces	Total	
Subject D							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Egg 1 (Last 3 of 7 days)	0.59	5.93	6.52	6.12	1.57	7.69	— 1.17
Milk (Last 3 of 7 days)	0.59	6.09	6.68	5.28	1.79	7.07	— 0.39
Egg 2 (Last 2 of 3 days)	0.59	5.93	6.52	5.32	1.24	6.56	— 0.04
Average of 2 egg periods	0.59	5.93	6.52	5.72	1.40	7.12	— 0.60
Subject R							
Egg 1 (Last 3 of 7 days)	0.59	5.93	6.52	5.16	1.50	6.66	— 0.14
Milk (Last 3 of 7 days)	0.59	6.09	6.68	4.72	1.60	6.32	+ 0.36
Egg 2 (Last 2 of 3 days)	0.59	5.93	6.52	4.70	1.50	6.20	+ 0.32
Average of 2 egg periods	0.59	5.93	6.52	4.93	1.50	6.43	+ 0.09
Replacement value of milk—subject D 100% subject R 100%							

Comparing the nitrogen balances of the single milk period with the average balances in the two adjacent egg periods reveals the fact that powdered whole milk has, for these two subjects, replaced whole egg as a source of protein completely and with something to spare. One does not wish to lay too much emphasis on only two cases, even though, as in this instance, the subjects seem to have cooperated perfectly.

The results are offered as indicating, though not proving conclusively, that at a higher level some proteins may make a better showing in terms of a given standard than at a lower level. Further results at the 6-gm. level will be published in the near future.

DIGESTIBILITY

For an accurate comparison of the digestibility of the different proteins, allowance should be made for the alimentary nitrogen of the feces. Mitchell ('24) and Heupke et al. ('33, '35) have shown that the coefficients of digestibility in which

TABLE 5

Average coefficients of digestibility

Food	*Apparent* %	*Estimated true,* %
Egg period 1	76 (66–87)	97 (93–100)
Powdered whole milk	80 (72–86)	99 (98–100)
Fresh milk	77 (71–84)	98 (91–100)
Powdered whole milk and yeast	69 (57–83)	93 (90– 96)
Fresh milk and yeast	73 (53–83)	96 (88–100)
Egg period 2	81 (73–91)	98 (97–100)
Egg and yeast		
Period 1	70 (56–83)	93 (78–100)
Period 2	70 (55–89)	94 (86–100)
Wheat endosperm		
Period 1	77 (69–82)	95 (87–100)
Period 2	80 (69–91)	96 (84–100)

this deduction has not been made are too low. The terms 'apparent' and 'true' or 'corrected' are used to differentiate between results for which this correction has not or has been made. The values calculated for this study belong to the former group. To estimate the true coefficient of digestibility a calculation of the alimentary nitrogen has been made according to Heupke, who found that for 20 gm. of total solids in the feces, originating from a simple mixed diet, roughly 1 gm. of nitrogen is of alimentary origin. The apparent coefficients and the estimated true coefficients are listed in table 5.

By either method of calculation, the nitrogen of dry milk or of fresh milk is absorbed to about the same extent. This is in accord with the work of London and his associates ('33) on dogs, showing for milk powder an assimilation value of 94.6% and for fresh milk of 96%. The substitution of yeast lowers the apparent coefficient of reconstructed dry milk and of egg. When an estimation is made of the true coefficient of digestibility, there is less difference between the periods with and without yeast than shown by the other set of values. There is no difference by either method of calculation between the absorption of milk compared with egg, when the averages of the two egg periods and the two milk periods are compared. The second wheat endosperm period shows a coefficient of digestibility equal to that of egg or milk.

Discussion

The conditions under which Sherman ('20) determined the efficiency of white bread in maintaining nitrogen equilibrium in man are quite similar to those of this study.

Over a 15-day period, with a total nitrogen intake of 6.0 to 6.09 gm. of which 96% came from bread, Sherman observed balances of —1.3, +0.2, —0.4, —0.2 and +0.2 for the five 3-day periods. In the second wheat endosperm period, on nitrogen intakes nearly 2 gm. less than that of Sherman, balances of —0.39, —0.51, —0.81, —0.92 and —1.2 were shown by five of the men subjects.

The experimental results on milk for the two women subjects compare quite favorably with the values reported for women subjects by Rose and MacLeod. For a period which was 5 days shorter, and with daily nitrogen intakes of from 1.5 to 2.5 gm. less than the subjects of Rose and MacLeod, negative balances of only —0.34 and —0.15 were observed, in contrast to the positive balances of +0.55 and +0.05 and +0.46 gm. of the earlier workers.

The superiority of egg protein to that of milk in lowering the negative nitrogen balance of human adults is demonstrated here for the first time. All of the ten subjects except one,

were accustomed to take from 1 to 2 pints of milk daily, so that the milk could not have been put to a disadvantage through imperfect digestion. In the second egg period, both women were in positive balance, and three of the men had negative balances ranging from —0.02 to —0.23 gm. These results are in agreement with those for adult and young growing rats, namely, that the biological value of egg protein is higher than that of milk (Sumner, '38).

Considered on the basis of nitrogen balance, it is doubtful if the dry milk is even slightly superior to fresh, pasteurized milk.

TABLE 6

Comparison of urinary and fecal N for yeast and non-yeast periods. Average daily with ranges

Period	NUMBER OF SUBJECTS	URINE N	FECAL N	FECAL TOTAL SOLIDS
		gm.	*gm.*	*gm.*
Milk [1]	10	4.12 (2.82–5.34)	0.79 (0.37–1.17)	18.13 (9.8–42.08)
Milk and yeast	9	4.11 (2.46–5.01)	1.21 (0.50–1.86)	21.51 (7.8–42.80)
Egg period 2	10	3.55 (2.23–3.99)	0.74 (0.23–1.11)	16.06 (11.3–30.6)
Egg and yeast, period 1	10	3.55 (1.90–4.38)	1.14 (0.70–1.92)	18.86 (7.3–26.8)
Egg and yeast, period 2	10	3.58 (2.01–4.19)	1.16 (0.41–1.81)	20.87 (11.8–30.7)

[1] Average for both dry and fresh milk.

An increase in total solids and in nitrogen in the feces is observed when 0.8 gm. of yeast nitrogen replaces an equal amount of either milk or egg nitrogen. The order of increase in total solids is from 3 to 4.5 gm. as shown in table 6.

Values for one subject are not included in the above averages, for milk and yeast, inasmuch as the total solids in his case were very much higher than for any of the other subjects, although his nitrogen values fell within the range found for the others.

If the average urinary nitrogen for all the subjects on milk or egg be compared with the corresponding yeast period, it will be seen that the amount excreted is unchanged. Never-

theless, in twelve instances out of twenty-nine there were slight increases in urinary nitrogen during the yeast periods. With the exception of one subject who jumped 0.71 gm. the variations (0.06 to 0.29 gm.) are insignificant. Fecal nitrogens were with two exceptions (decreases of 0.02 and 0.19) greater when the diet contained yeast.

The increment in fecal nitrogen between the yeast and the non-yeast periods for both milk and egg is 0.42 gm., an amount which is about half the nitrogen intake from yeast.

Pierce ('32) noticed this same relationship. When three cakes of yeast were superimposed on the control diet, the increase in fecal nitrogen was roughly half the amount supplied by the yeast. The corresponding increase in alimentary nitrogen calculated from the total solids according to Heupke, would amount approximately to 0.2 gm., or 50% of the total increase observed for fecal nitrogen. This is of course a rough calculation, but it would seem close enough to draw the conclusion that half of the extra fecal nitrogen in the yeast periods could be of body origin, inasmuch as the diets of the contrasted periods were of the same texture and composition with the exception of the added yeast.

However, this increase could also be due to unabsorbed yeast nitrogen. Kuen and Püringer ('34) contend that the nitrogen of bakers' pressed yeast, either fresh or killed by boiling, is poorly utilized by human subjects, only 52% being available to the organism. If these workers are correct in their assumption, the extra nitrogen of the stool could come entirely from unabsorbed yeast, as the 0.4 gm. is approximately half of the total nitrogen content of the ingested yeast.

The difference between the average balance for milk, and the combination of milk and yeast is —0.2 gm.; for egg, and egg and yeast —0.44 gm. This increase in the direction of an adverse nitrogen balance, is slightly less for milk than for egg. Moreover, it must not be overlooked that there was no significant increase in urinary nitrogen in any of the yeast periods except for one. This fact alone would point to a good utilization of the absorbed yeast nitrogen.

Theoretically, the ability of yeast to replace 0.8 gm. of either milk or egg nitrogen might be determined with greater clarity at the minimum requirement for nitrogen equilibrium. That all the subjects of this experiment received nitrogen in excess of their minimum requirement is undoubtedly true. Smith ('26) reports a value of 2.62 gm. nitrogen per day for urine and feces, Thomas ('09) of 2.2 gm., and Deuel et al. ('28) of 2.10 gm. for urine. If 1 gm. of nitrogen be added to this as representing the average fecal nitrogen excretion on a nitrogen-free diet, a total of slightly over 3 gm. is obtained. McClellan and Hannon ('32) were able to keep a diabetic subject in nitrogen equilibrium on an intake of 3.2 gm. of nitrogen per day. All these estimates are considerably lower than the amount of food nitrogen supplied to the men subjects in this study. Under the present circumstances therefore all that can be said with assurance is that the substitution of one-fifth of the nitrogen of milk or egg, at the 4-gm. level, with yeast nitrogen has a decidedly unfavorable effect on N-balance.

The apparent digestibility coefficients given by Atwater ('02) for egg, 97, dairy products, 97, and cereals, 85, are considerably higher than the values found in the present study, 81, 79 and 80, respectively. Murlin and Mattill ('38) found their apparent coefficients for cereals were lower than those commonly reported in the literature for human subjects. As the total nitrogen supplied by their diets amounted to approximately 6 gm., an amount considerably less than that employed by earlier workers, they concluded that the amount of protein in the diet influenced the coefficient of digestibility. The results of the present work on milk and egg are in keeping with this observation.

That the amount of nitrogen in the diet influences replacement values is strongly indicated by the work done at a 6.5 gm. intake. With nitrogen intakes of 3 gm. for the women and 4 gm. for the men the average replacement value of milk dropped to 86 in contrast with 100 at the higher level. The replacement value has been calculated also from the data of three subjects participating in the biological value study,

to be reported later, whose diets furnished either 3 or 4 gm. of nitrogen. Dry milk replaced egg flake 82, 87 and 95%, respectively. These figures fall within the range found for dry milk in the present study. This same effect may be shown from data on adult rats in which the proteins of milk and egg were reversed in consecutive periods during the determination of the biological value (Sumner, '38). The replacement value has been computed for these animals at both the 5 and the 8% levels, and as for the human subjects, a higher value is obtained with the greater nitrogen intake. These results are in accord with the view that a higher intake of protein

TABLE 7

Replacement value at different levels of intake in human subjects and rats

		Dry milk
Subject	N intake	R.V., %
Young men	6 gm. N/day	100 [2]
Young men	3–4 gm. N/day [1]	85 (81– 95)
Adult rats	8% level	96 (88–100)
	5% level	81 (55–100)

[1] Equal to 3.3% of total calories.

[2] Calculation of 'biological values' on the assumption that egg protein is 100 gives a similar difference between the two levels of intake.

may conceal the fact that it is low in some essential amino acid, which for milk is cystine (Mitchell and Beadles, '30). There is also fair agreement in the results for the two species at either the higher or lower plane, as the accompanying summary shows.

The average replacement values are more nearly identical for the two species than the figures for biological values. At the higher level of nitrogen intake, milk is able to replace egg nitrogen 100% in human nutrition, compared to 96% for adult rats. At the lower level, the replacement value is 84 for the human subjects and 81 for the rats.

CONCLUSIONS

Egg protein is superior to milk protein in maintaining nitrogen balance of adult human subjects.

Powdered milk and fresh milk replace egg nitrogen to about the same extent.

Wheat endosperm used with a small amount of 4X cream replaces egg nitrogen fully as well as milk.

When yeast is substituted for a fraction of the milk or egg nitrogen, an increased negative balance results in the majority of the subjects.

The nitrogen and total solids of the feces increase during the yeast periods. There is no corresponding increase in the urinary nitrogen.

The apparent coefficients of digestibility are of the same order of merit for egg, wheat endosperm and fresh or dry milk.

The replacement value of milk, using egg as the standard, is similar for rats and men.

The proteins of milk and of egg are equally well assimilated by human subjects or by rats, whether measured by the apparent or true coefficient of digestibility.

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CONCERNING THE TOXICITY OF VITAMIN A

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Several investigators have described a pathological condition which they called hypervitaminosis A. Von Drigalski and Laubman ('33) fed six rats 1 cc. of an oil concentrate of vitamin A. These rats died in from 5 to 14 days, and post mortem examination showed glomerular nephritis with calcification to which the death of the animals was attributed. Pathologic changes on a smaller scale were noted in the spleen, liver and testicles. Collazo and Rodriguez ('33) also fed several 50-gm. rats a vitamin A concentrate, Vogan, prepared by E. Merck of Darmstad, Germany. The rats were fed 0.5 cc. daily, or a dosage of 20,000 units of vitamin A. The outstanding features of the disease produced in about 10 days were failure to grow, inflammatory changes in the eyes with exophthalmos and changes in the bones resulting in spontaneous fractures. Bumskov and Seeman ('33) obtained similar results in eleven rats from the administration of Vogan, and somewhat similar observations have been published by Harris and Moore ('29) and others.

The results obtained were attributed to vitamin A by these authors chiefly because the administration of similar amounts of sesame oil and other vegetable oils containing no vitamin A are harmless, and because several observers had found that vitamin A was destroyed by ultra-violet irradiation and that the toxicity of the oil was also lost. Thus von Drigalski and Laubman ('33) submitted the toxic Vogan to ultra-violet

irradiation, and finding that this destroyed the toxic power of this concentrate, concluded that this proved that the toxic effect was produced by vitamin A. This conclusion does not necessarily follow because the toxic effect may have been due to some other constituent in the oil also destroyed by ultra-violet radiation. Peacock ('26) showed that irradiated cod liver oil lost its normal fluorescence in addition to the loss of vitamin A and that these two phenomena were different and independent. Irradiation of such an oil also produced vitamin D which is supposed to have an antagonistic action to vitamin A. For these reasons, the above experiments, performed moreover on very small series of rats, hardly prove that the toxicity of a complicated fish liver oil concentrate is caused by its vitamin A content. It is also known that the feeding of carotene, the pro-vitamin A, is entirely harmless, a fact that we have confirmed by feeding several young rats 40,000 international units both alone and in solution in vegetable oil for 100 days without ill effect.

Since Vogan is unobtainable in this country, we obtained a vitamin A concentrate from the British drug houses, having a Carr-Price blue value of not less than 50,000. Lathbury ('34) has shown that there is a constant ratio between the results of the biological, chemical and physical methods of testing purified vitamin A concentrates. Half a cubic centimeter of this concentrate was administered to 50-gm. rats daily, but this preparation was so objectionable that rats so treated refused any food and died of starvation in 4 days.

In the following experiments we have used 140 rats, of which 127 form the basis of this report, the remainder being various controls. These experiments were all performed with oil from the liver of the jewfish (Stereolepis gigas), which is the richest source of vitamin A known at present, being many times more potent than concentrates from ordinary oils. All of the oil so used was furnished us through the courtesy of Dr. Charles E. Bills, director of the Research Laboratory of Mead Johnson and Company.

Technic. Healthy male rats of the Wistar strain and of 50 gm. weight were used in all experiments, except that females were introduced at times to determine breeding capacity. The diet fed was Purina dog chow, which is a complete diet for rats. Stock rats so fed grew and bred normally. This diet was used for its convenience in weighing in order to determine the amount eaten daily. The oils used were administered daily by means of a pipette, and dosage was kept at 0.5 cc., changes in concentration being effected by proper dilution with oil of sesame, shown in controls to be inert. The vitamin A potency of the oil was determined by vitameter readings, and it had been planned to dose all rats with multiples of 25,000 international units. Unfortunately, it was found later that owing to some unknown constituent in the jewfish liver oil, the vitameter readings were about one-third higher than the results of the biological test. In experiments performed later, 100,000 units by biological test were used.

The experiments were all continued for 100 days unless death occurred earlier. It was assumed that if rats continued to grow normally for 100 days, the oil administered was non-toxic. A post mortem examination was made on all rats that died as a result of the treatment, and later all the surviving rats were killed not only for post mortem examination, but to determine the vitamin A content of their livers.

The number of units stored in the liver was determined by the blue reaction with antimony trichloride. The antimony trichloride is used in a 30% chloroform solution (weight in volume). The liver to be tested was dissolved in a 10% solution of potassium hydroxide; this solution was extracted in a separatory funnel with ether; the ether evaporated, and the residue redissolved in chloroform. To 0.2 cc. of this chloroform solution, which was usually made up to 500 cc., 2 cc. of the antimony trichloride was added, and the resultant blue color matched against the standard glasses of a Lovibond tintometer, in accordance with the method described in "The Vitamins: Symposium on the present Status of the Knowledge of Vitamins." J. Am. Med. Assn., April 23 to August 6, 1932.

We were unable to confirm the pathological findings of von Drigalski and Laubman ('33). At post mortem, all the organs except the bones appeared normal grossly. Microscopic sections showed that the stomach and intestines, the testes,

adrenals, thyroid and parathyroid were normal. There were often mild congestion of the lungs, marked congestion and some cloudy swelling and, at times, fatty infiltration of the liver, moderate congestion and cloudy swelling of the tubules of the kidney, the glomeruli appearing normal, and a spleen slightly congested. In some cases there appeared to be some swelling of the heart muscle fibers, and a loss of staining of their nuclei. While these findings were by no means invariable, they were frequent enough to indicate that death was probably caused by an insufficiency of the cardiac muscle with accompanying congestion of the abdominal viscera.

The symptoms agreed very well with those described by Collazo and Rodriguez ('33) except that they were only manifested by some of our rats. They included a diminished growth curve, hemorrhages from the eyes often with exophthalmos, hemorrhages from the nose, rales in the chest, and the bone lesions. The bone lesion is rarefaction with a pronounced tendency to spontaneous fracture. If the animal lived, the fractures healed in spite of the continuance of the large dosage of vitamin A.

In the following tables presenting the results of our experiments, both vitameter readings and the results of the biological test are given. Since three separate lots of jewfish liver oil were used, there are three series of experiments tabulated.

Series 1. This lot of oil contained 550,000 international units of vitamin A per cubic centimeter (vitameter) and 4500 units of vitamin D.

Results. Series 1

RATS	DOSAGE I.U.A.[1]	AVERAGE FINAL WEIGHT	REMARKS
1– 4	25,000	259	Normal except some decrease in weight
5– 8	50,000	241	One rat died in 40 days. All had fractures
9–20	100,000	45	No rat lived 100 days. Fractures and hemorrhages.

[1] By biological test these amounts were 16,000, 33,000 and 66,000 international units, respectively.

Series 2. This lot of oil contained 663,000 international units of vitamin A per cubic centimeter (vitameter), and 4500 units of vitamin D.

Results. Series 2

RATS	DOSAGE I.U.A.[1]	AVERAGE FINAL WEIGHT	REMARKS
1– 4	25,000	250	Normal
5– 8	50,000	194	Fractures in two rats. All lived 100 days
9–16	100,000	127	Two rats lived 100 days. Six had fractures

[1] By biological test these amounts were 16,000, 33,000 and 66,000 international units, respectively.

Series 3. This lot of oil contained 600,000 international units of vitamin A per cubic centimeter (vitameter) and 5000 units of vitamin D.

Results. Series 3

RATS	DOSAGE I.U.A.[1]	AVERAGE FINAL WEIGHT	UNITS VITAMIN A PER GRAM LIVER	REMARKS
1– 4	50,000	319	1766	Normal. All rats lived 100 days
5–12	100,000	123	1704	Three rats lived 100 days. Two rats had fractures

[1] By biological test these amounts were 33,000 and 66,000 international units, respectively.

Series 4. One liter of jewfish oil having 530,000 international units of vitamin A per gram was submitted to molecular distillation by Dr. K. Hickman of the research laboratories of the Eastman Kodak Company. Six lots of oil were used as follows:

1. The original oil as a control containing 530,000 I.U. vitamin A per gram
2. Distilled fraction 1 containing 515,000 I.U. vitamin A per gram
3. Distilled fraction 2 containing 865,000 I.U. vitamin A per gram
4. Distilled fraction 3 containing 805,000 I.U. vitamin A per gram
5. Distilled fraction 4 containing 248,000 I.U. vitamin A per gram
6. The residue from distillation containing 53,000 I.U. vitamin A per gram

In the first five cases, the oil was so diluted with sesame oil that each 0.5 cc., the daily dosage, contained 100,000 international units of vitamin A. In the case of the residue, 2 cc. of undiluted oil were administered daily.

Results. Series 4

RATS	DOSAGE I.U.A. 100,000	AVERAGE FINAL WEIGHT	UNITS VITAMIN A PER GRAM LIVER	REMARKS
1– 4	Original oil	87	818	Only one lived 100 days. Fractures
5– 8	Fraction 1	135	4722	Three rats lived 100 days. No fractures
9–12	Fraction 2	97	3250	One rat lived 100 days. No fractures
13–16	Fraction 3	89	3250	One rat lived 100 days. Three had fractures
17–20	Fraction 4	38		Average life was 12 days. Hemorrhages
21–24	Residue	272	2199	All lived 100 days. Normal

Fraction 1 and the residue were less toxic than the other fractions. To be sure that this was the fact, each was repeated on a new lot of four rats, with precisely the same result. One female was introduced in the four receiving the residue, and nine young were born on the fifty-second day of the experiment. All nine lived.

Since jewfish oil was undoubtedly toxic in amounts equivalent to 100,000 international units of vitamin A, we next endeavored to ascertain whether this toxic effect was due to vitamin imbalance. For this purpose we used lot three of the jewfish oil, which was non-toxic in doses of 50,000 international units, and decidedly toxic in doses of 100,000 units (vitameter reading). Other vitamins were administered in addition. Vitamin D was administered from appropriate dilutions of a solution of irradiated ergosterol containing 1,000,000 units per cubic centimeter. Vitamin B_1 was used in two forms: an adsorbate received through the courtesy of the Eli Lilly Company, and crystalline vitamin synthesized and furnished us through the kindness of Merck and Company.

Vitamin C was also the synthetic product, furnished by Merck and Company. The results in the following table should be compared with the results in series 3 in which the same lot of jewfish oil was used alone.

Results from addition of other vitamins

RATS	DOSAGE	AVERAGE FINAL WEIGHT	UNITS VITAMIN A PER GRAM IN LIVER	REMARKS
1– 4	50,000A + 50,000D	44		Average life 27 days. No fractures
5– 8	50,000 + 25,000D	209	2731	Three lived 100 days. One rat had fractures
9–12	100,000A + 100,000D	39		Average life 10 days. No fractures
13–20	100,000A + 50,000D	190	2093	Five lived 100 days. No fractures
21–24	100,000A + 500 B_1	54		None lived 100 days. Average life 44 days. All had fractures
25–36	100,000A + 50,000D + 500 B_1	84	2750	Three lived 100 days. Two had fractures
37–48	100,000A[1] + 5 mg. ascorbic acid	241	2560	All lived 100 days. All normal and one raised ten young

[1] One hundred thousand international units vitamin A per cubic centimeter by biological test.

DISCUSSION

The term toxicity means poisonous. Many food materials such as sodium chloride or iodine can become deleterious in large quantities, but they are not called poisons. Neither should fish oils be called poisons because they are injurious when fed in enormous quantities. But some term is necessary to refer to this deleterious action in these experiments, and we have used toxicity, with the understanding that in the usual doses the various ingredients of such oils may be necessary in the diet.

The question as to the toxicity of large doses of vitamin A cannot be regarded as completely settled by these experiments, and the complete answer must await the isolation of

vitamin A in pure form. Nevertheless, several facts appear to indicate that the toxicity of jewfish liver oil is due to some constituent of the oil other than vitamin A. In the same dosage of vitamin A, the toxicity varies for each lot of oil. According to the European observers, Vogan is highly toxic, killing rats in about 2 weeks in doses containing 20,000 units of vitamin A. No sample of jewfish liver oil was toxic in less than 33,000 international units daily and the toxicity of lot 3 of the jewfish liver oil only commenced at 66,000 international units daily. Further, our 50-gm. rats receiving 33,000 units lived 100 days as a rule. It would seem that if vitamin A is the toxic factor, the toxicity of different samples of fish liver oil should be proportional to their vitamin A content. This is certainly not the case.

Still more significant is the fact that when lot 4 of the jewfish liver oil was submitted to molecular distillation, the toxic substance was separated from vitamin A. In the dosage of 100,000 international units of vitamin A daily, fraction four killed all rats in from 9 to 26 days, while three out of four rats receiving the same dosage of vitamin A from fraction one, lived 100 days. Finally, the four rats receiving 100,000 units of vitamin A from the residue from the distillation not only lived 100 days, but grew normally and were capable of bearing and rearing young. This result cannot be due to vitamin D which was only present in the original oil in doses of 5000 units. If it had all been distilled in one fraction, a most improbable supposition, it would have been quite insufficient to balance 100,000 units of vitamin A. We have shown that about 50,000 units of D are required for this purpose. We may conclude from this experiment, which was repeated with the same result, that if vitamin A is toxic, it must be given in higher dosage than 100,000 international units daily to 50-gm. rats, and that at least the greater part of the toxicity of the various fish liver oils must be due to some other factor than vitamin A. Since there is an increase of one-third in the vitameter reading over the result of the biological test as applied to jewfish liver oil, this discrepancy

is due to some unknown constituent in this particular oil. It is possible that this unknown constituent is the toxic fraction.

The greater part of vitamin A administered in these large doses must be either excreted or broken down in the body. In the course of 100 days at a dosage of 100,000 units daily, 10,000,000 units have been given. The maximum amount found in any liver per gram was 5000 units, which, with a 7-gm. liver was sufficient vitamin A to last this rat many years. The largest total amount found in the liver of any rat was rat no. 3 of series 3, with 2794 units per gram, which with 17 gm. of liver equalled 47,550 units. While lesser amounts are stored in other organs, only about 1 day's dosage, or one-hundredth part of the 10,000,000 units administered, can be accounted for. This fact is more obvious in our experiments than in those of other observers, because we have used much larger dosage, and kept our rats for much longer periods. No vitamin A is excreted in the urine, and of several rats tested, only from 500 to 600 units were excreted daily in the feces. Since the rat eliminates so little vitamin A, it seems certain that most of the vitamin A administered is destroyed in the body, and this constitutes another argument against the toxicity of this vitamin.

The neutralization of the toxic effect of fish liver oil by the administration of other vitamins has been suggested by numerous observers. Bell, Gregory and Drummond ('33) and others believe that the disturbance in the growth of rats that occurs when the diet contains a considerable percentage of fish liver oil may be compensated by an increased amount of the vitamin B complex. So far as our experiments with jew-fish liver oil are concerned, the administration of 500 international units of B_1 daily appeared to be not only of no value, but to be positively injurious. McHenry ('36) has shown that the addition of vitamin B_1 to rats on a basal diet practically doubles the percentage of fat in the liver. Whether used alone, or in combination with vitamin D, and whether used as an adsorbate or as crystalline vitamin, the results were poorer than control series without B_1. Of the rats receiving vitamin

A at 66,000 units, with vitamin D and vitamin B_1, only three out of twelve lived 100 days; but on the same dosage of A and D without B_1, five out of eight lived 100 days.

Thoenes ('35) and others have asserted that vitamin A and vitamin D are physiologically antagonistic; that is, that vitamin D is antagonistic to vitamin A concentrates. Our experiments afford some confirmation of this view, or at least that administration of considerable amounts of vitamin D relieves the toxic effects caused by jewfish liver oil. When the unitage of vitamin D exceeded that of vitamin A, the combination was far more toxic than the same amount of vitamin A alone. But vitamin D was of distinct benefit when its dosage was somewhat less than that of the vitamin A of jewfish oil. Thus when jewfish liver oil was given so as to administer 66,000 international units of vitamin A and 50,000 units of vitamin D, five out of eight rats lived 100 days; whereas, with the same lot of jewfish liver oil alone, administered in the same dosage, only two out of eight lived 100 days. Perhaps a still better proportion between the vitamin A containing fish liver oil and vitamin D dosage is possible, since we only tried two proportions.

The most striking result of these experiments, and one that so far as we are aware has never been suggested, is the preeminent protective action of pure crystalline ascorbic acid or vitamin C. Five milligrams of this vitamin given daily is apparently sufficient to protect all rats receiving such doses of jewfish oil as those containing 100,000 international units of vitamin A. Eleven out of twelve rats receiving such dosage of jewfish liver oil not only lived 100 days, but many of them grew normally. The only rat of this series that died earlier had a severe intercurrent infection which probably excludes it from consideration. No other dosage of vitamin C was used, and it is possible that smaller doses would suffice, since ½ mg. daily should be approximately the amount required by an animal the size of a rat. On the other hand, larger doses may give a better result, as indicated by the fact that several of these rats developed fractures.

Since the rat is known to be capable of synthesizing vitamin C and is not susceptible to scurvy, it is difficult to explain this action of vitamin C. A possible explanation is that vitamin C destroys the toxic factor and that all the vitamin C synthesized by the rat is used up in this process, leaving it susceptible to scurvy. Scurvy of the rat has never been described, but the symptoms produced by fish liver oils are not unlike scurvy; they include failure to grow, hemorrhages, particularly from the eyes and nose but to some extent from other mucous membranes, and abnormal rarefaction and fragility of the bones.

In a few rats, the urine was collected and titrated with 2,6-dichlorophenol indophenol. After administration of jewfish liver oil equivalent to 100,000 units of vitamin A for about 3 weeks, the excretion of vitamin C decreased to the vanishing point, but reappeared in the urine in considerable amounts after the daily administration of 5 mg. of crystalline ascorbic acid. While this is suggestive, since the physiological action of vitamin C is quite unknown, the correct explanation of its remedial action for jewfish liver oil must await further experimentation.

CONCLUSIONS

If vitamin A is ever toxic, it is in doses in excess of 100,000 international units daily for 50-gm. rats.

The greater part of the vitamin A administered in such large dosage is destroyed in the body of the rat.

In addition to vitamin A, fish liver oils contain a toxic principle, at present not identified. The toxicity of various samples of these oils and their concentrates varies greatly for rats.

Vitamin B_1 was of no value in counteracting the toxicity of jewfish liver oil for rats.

Vitamin D in proper dosage at least partially counteracts this toxicity of jewfish liver oil as administered to rats.

Ascorbic acid (crystalline vitamin C) in doses of 5 mg. daily almost completely counteracted the toxicity of jewfish liver oil administered to rats.

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ENVIRONMENTAL TEMPERATURE AND 'RAT ACRODYNIA'[1]

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Rats kept on a vitamin B-free synthetic diet containing extracted casein, rice starch or cane sugar, melted butterfat, cod liver oil, and salt mixture (McCollum 185) and supplemented with vitamin B_1 and riboflavin, manifest a symmetrical dermatitis in from 4 to 15 weeks (on an average in 7 weeks) (György, '35 a). These skin manifestations, termed recently 'rat acrodynia' (Birch, György and Harris, '35), are due to a diet deficient in a specific constituent of the vitamin B_2 complex, named vitamin B_6.

In observations here recorded it has been shown that environmental factors, such as temperature, may profoundly influence the appearance of symptoms of vitamin B_6 deficiency. In summer on days when the outdoor air was very warm, the temperature inside the animal room could not be controlled, and it ranged often for weeks between 80° and 100° F. During the same periods the incidence of acrodynia in the rats was distinctly on the decline but became normal again with lower temperature conditions.

The reverse, that is, acceleration in the production of acrodynia, was achieved by exposing rats to cold temperature. In these experiments animals of one litter were divided into two groups. Between 9 A.M. and 5 P.M. both groups were kept

[1] For earlier papers in this series, see Literature Cited as follows: György, '35 a; György, '35 b; György, '35 c; Birch, György and Harris, '35; and Birch and György, '36.

under identical experimental conditions at normal room temperature. At 5 P.M. one group was placed daily in a refrigerator room, with temperature at about 40° F., until 9 A.M. the next day, while the control group was allowed to remain in the room where temperature conditions were unchanged. During the first few days of each experiment the rats were not exposed suddenly for the whole period of 16 hours but were exposed gradually, for instance, on the first day for 1 hour, on the second for 3 hours and on the third for 6 hours. By means of this technic, difficulties in acclimatization of the rats to extended cold periods were avoided.

In all these experiments a very high food intake was encountered consistently in the rats exposed to cold, without a corresponding increase in weight, whereas the control rats, on the same deficient diet, consumed less food, with, at least temporarily, better growth. Table 1 shows selected data obtained in the first experiment which comprised eight rats from one litter, four of which were exposed to cold. In only one (rat 6948) of these four was growth of about the same proportion as in the control animals; in the other three, growth was almost completely inhibited.

Far more important than this finding is the fact that, again with the exception of rat 6948, acrodynia-like symptoms developed after a much shorter period in the three other rats exposed to cold than in the control animals. The first manifestations of acrodynia were noticed in these four rats after 4 (rat 6939), 9 (rat 6943) and 5 weeks (rat 6945), but in the control group only at the end of 19 (rat 6940), 27 (rats 6942 and 6946) and even 34 weeks (rat 6944). Moreover, in rat 6943 the skin manifestations disappeared as soon as exposure to cold was discontinued and the rat was put back permanently in the normal temperature room with no further change in experimental and, particularly, in nutritional conditions.

In spite of its early appearance, the acrodynia in the rats exposed to cold showed a slower course, as well as some other peculiarities, such as prevalence of erosions of the lips and angles of the mouth corresponding to pathological conditions

TABLE 1

Experiment 1

RAT NO.	DATE	WEIGHT	EXPERIMENTAL CONDITIONS		SKIN MANIFESTATIONS OF B_2 DEFICIENCY
			Nutrition	Temperature	
		gm.			
6939 ♀	5/21/36	39	B_1 + lactoflavin	Refrigerator	
	6/18	45	B_1 + lactoflavin	Refrigerator	Beginning acrodynia (perlèche)
	7/16	41	+ 1 cc. Peters' eluate	Refrigerator	Acrodynia
	8/27	66	+ 1 cc. Peters' eluate	Refrigerator	Improving
	9/ 3	71	+ 1 cc. Peters' eluate	Refrigerator	Acrodynia cured
6943 ♀	5/21/36	38	B_1 + lactoflavin	Refrigerator	
	6/18	44	B_1 + lactoflavin	Refrigerator	
	7/23	50	B_1 + lactoflavin	Refrigerator	Beginning acrodynia
	7/30	52	B_1 + lactoflavin	Refrigerator	Acrodynia
	8/ 6	63	B_1 + lactoflavin	Room	Acrodynia
	8/13	72	B_1 + lactoflavin	Room	Spontaneous recovery
	8/27	81	B_1 + lactoflavin	Room	Acrodynia cured
6945 ♂	5/21/36	36	B_1 + lactoflavin	Refrigerator	
	6/25	40	B_1 + lactoflavin	Refrigerator	Beginning acrodynia
	7/ 2	40	B_1 + lactoflavin	Refrigerator	Acrodynia
	8/ 6	43	+ Peters' eluate	Refrigerator	Acrodynia
	8/13	53	+ Peters' eluate	Refrigerator	Acrodynia improving
	8/27	70	+ Peters' eluate	Refrigerator	Acrodynia cured
6948 ♂	5/21/36	36	B_1 + lactoflavin	Refrigerator	
	6/18	50	B_1 + lactoflavin	Refrigerator	
	7/23	71	B_1 + lactoflavin	Refrigerator	
	8/20	84	B_1 + lactoflavin	Refrigerator	
	9/24	95	B_1 + lactoflavin	Refrigerator	
	10/29	93	B_1 + lactoflavin	Refrigerator	
	11/19	92	B_1 + lactoflavin	Refrigerator	Acrodynia
6940 ♂	5/21/36	36	B_1 + lactoflavin	Room	
	6/25	62	B_1 + lactoflavin	Room	
	7/30	83	B_1 + lactoflavin	Room	
	9/ 3	90	B_1 + lactoflavin	Room	
	10/29	102	B_1 + lactoflavin	Room	
	11/26	92	B_1 + lactoflavin	Room	Beginning acrodynia
6942 ♀	5/21/36	40	B_1 + lactoflavin	Room	
	6/18	65	B_1 + lactoflavin	Room	
	7/23	85	B_1 + lactoflavin	Room	
	8/27	103	B_1 + lactoflavin	Room	
	10/ 1	105	B_1 + lactoflavin	Room	
	11/ 5	114	B_1 + lactoflavin	Room	
	11/26	99	B_1 + lactoflavin	Room	Beginning acrodynia
	12/24	90	B_1 + lactoflavin	Room	Acrodynia
6944 ♂	5/21/36	42	B_1 + lactoflavin	Room	
	6/25	69	B_1 + lactoflavin	Room	
	7/30	84	B_1 + lactoflavin	Room	
	9/ 3	95	B_1 + lactoflavin	Room	
	10/ 8	108	B_1 + lactoflavin	Room	
	11/12	117	B_1 + lactoflavin	Room	
	12/10	103	B_1 + lactoflavin	Room	
	12/31	102	B_1 + lactoflavin	Room	No acrodynia
6946 ♀	5/21/36	32	B_1 + lactoflavin	Room	
	6/25	63	B_1 + lactoflavin	Room	
	7/30	80	B_1 + lactoflavin	Room	
	9/ 3	77	B_1 + lactoflavin	Room	
	10/ 1	75	B_1 + lactoflavin	Room	
	11/ 5	78	B_1 + lactoflavin	Room	
	11/26	77	B_1 + lactoflavin	Room	Beginning acrodynia

seen in man in angular stomatitis, or the so-called perlèche of French writers, whereas involvement of the extremities ensued at a relatively later stage of the disease.

Acrodynia in the rats exposed to cold was cured by treatment with Peters' eluate (Kinnersley, O'Brien, Peters and Reader, '33), as seen in table 1, rat 6939—a preparation which is rich in vitamin B_6 and which at the same time stimulated growth. Thus it is demonstrated that the effect of Peters' eluate here is analogous to its effect under normal conditions. The gain in weight, however, remained far behind that observed when an identical dosage was used under normal conditions.

The findings and conclusions resulting from the first test were duplicated and substantiated in four additional experiments, one of which is summarized in table 2. The average number of weeks required for production of acrodynia in the two groups of rats in these experiments was as follows:

	Number of rats	*Average number of weeks required in rats exposed to cold*	*Average number of weeks required in control rats*
2nd experiment	6	4	9
3rd experiment	13	5	15
4th experiment	8	7	9¼
5th experiment (table 2)	11	3	8

Similar results were obtained in several other tests not recorded here. They leave no doubt as to the validity of the conclusion that low temperature seems to be particularly conducive to production of acrodynia in rats kept on a vitamin B_6 deficient diet.

The early appearance of acrodynia-like symptoms in rats exposed to cold, as well as the almost complete suppression of growth in the same rats, is not due to some unspecific effect of cold temperature but can be regulated by diet. This fact became evident in experiments in which the vitamin supplement to the basal diet consisted not only of vitamin B_1 plus riboflavin but also of a vitamin B_6 preparation in the form of Peters' eluate, as in experiments 4 and 5 (compare table 2).

TABLE 2

Experiment 5

RAT NO.	DATE	WEIGHT	EXPERIMENTAL CONDITIONS		SKIN MANIFESTATIONS OF B_6 DEFICIENCY
			Nutrition	Temperature	
		gm.			
7338 ♀	9/24/36	27	B_1 + lactoflavin	Refrigerator	
	10/15	30	B_1 + lactoflavin	Refrigerator	Beg. acrodynia (perlèche)
	10/29	28	B_1 + lactoflavin	Refrigerator	Acrodynia
	10/31	28	B_1 + lactoflavin	Refrigerator	Death
7339 ♂	9/24/36	25	B_1 + lactoflavin	Refrigerator	
	10/15	29	B_1 + lactoflavin	Refrigerator	Beg. acrodynia (perlèche)
	10/29	27	B_1 + lactoflavin	Refrigerator	Acrodynia
7340 ♂	9/24/36	26	B_1 + lactoflavin + B_6	Refrigerator	
	10/22	70	B_1 + lactoflavin + B_6	Refrigerator	
	11/26	103	B_1 + lactoflavin + B_6	Refrigerator	
	12/24	120	B_1 + lactoflavin + B_6	Refrigerator	
	1/14	134	B_1 + lactoflavin + B_6	Refrigerator	
7341 ♂	9/24/36	27	B_1 + lactoflavin + B_6	Refrigerator	
	10/22	69	B_1 + lactoflavin + B_6	Refrigerator	
	11/19	89	B_1 + lactoflavin + B_6	Refrigerator	
	12/17	116	B_1 + lactoflavin + B_6	Refrigerator	
	1/14	132	B_1 + lactoflavin + B_6	Refrigerator	
7342 ♂	9/24/36	27	B_1 + lactoflavin	Refrigerator	
	10/15	32	B_1 + lactoflavin	Refrigerator	Beg. acrodynia (perlèche)
	10/29	32	B_1 + lactoflavin	Refrigerator	Acrodynia
	11/12	32	B_1 + lactoflavin	Room	Acrodynia
	12/ 3	42	B_1 + lactoflavin	Room	Acrodynia
7343 ♀	9/24/36	28	B_1 + lactoflavin	Refrigerator	
	10/22	34	B_1 + lactoflavin	Refrigerator	Beg. acrodynia
	11/ 5	33	B_1 + lactoflavin	Refrigerator	Acrodynia
	11/12	31	B_1 + lactoflavin	Room	Acrodynia
	12/ 3	26	B_1 + lactoflavin	Room	Acrodynia
7344 ♀	9/24/36	30	B_1 + lactoflavin	Room	
	10/22	43	B_1 + lactoflavin	Room	
	11/26	45	B_1 + lactoflavin	Room	Acrodynia
7345 ♀	9/24/36	29	B_1 + lactoflavin	Room	
	10/22	44	B_1 + lactoflavin	Room	
	11/19	41	B_1 + lactoflavin	Room	Acrodynia
7346 ♂	9/24/36	26	B_1 + lactoflavin + B_6	Room	
	10/22	65	B_1 + lactoflavin + B_6	Room	
	11/19	110	B_1 + lactoflavin + B_6	Room	
	12/17	122	B_1 + lactoflavin + B_6	Room	
	1/14	133	B_1 + lactoflavin + B_6	Room	
7347 ♂	9/24/36	28	B_1 + lactoflavin + B_6	Room	
	10/22	74	B_1 + lactoflavin + B_6	Room	
	11/19	122	B_1 + lactoflavin + B_6	Room	
	12/17	157	B_1 + lactoflavin + B_6	Room	
	1/ 7/37	169	B_1 + lactoflavin + B_6	Room	
7348 ♀	9/24/36	26	B_1 + lactoflavin	Room	
	10/22	39	B_1 + lactoflavin	Room	
	11/19	42	B_1 + lactoflavin	Room	Beg. acrodynia
	12/10	38	B_1 + lactoflavin	Room	Acrodynia

Addition of vitamin B_6 prevented production of the skin manifestations and also stimulated growth which could be considered satisfactory, although the growth was not so vigorous as that seen in the control rats kept at room temperature (table 2). The conclusion drawn is, therefore, warranted. The deleterious effect of environmental cold temperature on rats that have received only vitamin B_1 and riboflavin of the constituents of the vitamin B complex can be neutralized by the addition of vitamin B_6 or some other constituents of the vitamin B_2 complex present, for example, in Peters' eluate.

DISCUSSION

Temperature of the environment has not received much attention in vitamin research until the present time. Tourtellotte and Bacon ('35) stated that increased severity of rickets resulted in vitamin D test animals when they were subjected to laboratory temperature of 80° F. or above. In a recent paper by Guerrant, Dutcher and Crowthers ('37) these results have not been confirmed. Other than these papers there does not seem to be any pertinent work dealing with the problem of environmental temperature. And the papers mentioned do not cover the whole ground, since they are confined to the effect of normal and high temperatures and do not consider low temperature.

Recently Manville and Chase ('37) have pointed out that the ability of anemic rats to withstand low temperatures progressively decreases as the severity of the anemia increases. In these rats the anemia was apparently due mainly to lack of some factor of the vitamin B complex not present in yeast. But no mention is made of whether or not low temperature in itself enhances the production of anemia under these special nutritional conditions. In view of the depressing influence of cold environmental temperature on the bone marrow in rats, shown by Huggins and Blocksom ('36), one would suspect that the same physical factor should at least accelerate the production of anemia that follows panmyelophthisis in rats (György, Goldblatt, Miller and Fulton, '37). Contrary to these expectations, it was found in the experiments being discussed in the

present paper, that of thirty-four rats which for varying periods up to 26 weeks had been exposed daily between 5 P.M. and 9 A.M. to cold temperature only one animal developed anemia.

It is well known that in the absence of the antineuritic vitamin B_1 the body temperature may drop considerably and that animals kept on a diet deficient in vitamin B_1 must suffer from a cold environment much more than animals that have the ability to maintain a normal body temperature. In the experiments presented here, the vitamin B_1 intake was satisfactory and the body temperature was well regulated even during exposure of the rats to cold environment.

In order to explain the accelerating effect of cold environment on production of acrodynia in rats, therefore, there are only two possibilities to be taken into consideration. Either the need for vitamin B_6 is greater in rats subjected to cold temperature or, inasmuch as the basal diet in these experiments was deficient not only in B_6 but in several other constituents of the vitamin B_2 complex as well, the cold temperature may have turned the 'concurrence of vitamin deficiency diseases' in favor of the vitamin B_6 deficiency and suppressed the manifestations of other deficiencies more than is usually the case at normal temperature. Which of these alternatives is the true explanation is a question that can be settled only when a diet becomes available that is free from vitamin B_6 alone.

These observations on the special effect of cold environment on acrodynia-like skin manifestations in rats are not without possible clinical implications. In the first place one is reminded of the striking similarity between this disease in the rat and the well-known syndrome of chilblain in man. This similarity suggests the following questions: 1) Is not the susceptibility to chilblain a function of vitamin B_6 reserves in the body? 2) Would not medication with vitamin B_6 concentrates be of avail in the prevention of chilblain?

In the second place, the peculiar angular stomatitis (perlèche) which apparently is fairly common in British India

should be mentioned in this connection. The description of this disease given recently by Aykroyd and Krishnan ('36) shows that it closely resembles the first manifestations of acrodynia seen in the rats exposed to cold environment in the experiments discussed in the present paper. Aykroyd and Krishnan have emphasized the fact that the etiology of the human disease lies in a nutritional deficiency and the probability that it may be related to the lack of a factor of the vitamin B_2 complex, in their opinion, riboflavin. In the light of the present experimental investigations it would seem that the etiology of the human disease should rather be correlated to vitamin B_6 deficiency. Final clarification of this question rests with the outcome of special therapeutic trials.

SUMMARY

In rats exposed to a cold environment with temperature of 40° F., acrodynia appears earlier than in control animals kept in a room at normal temperature. Peters' eluate cures and prevents acrodynia in rats exposed to cold environment in the same way as in the control animals. The possible clinical implications of this finding are discussed.

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THE AVAILABILITY OF CALCIUM IN SPINACH, IN SKIM MILK POWDER, AND IN CALCIUM OXALATE [1]

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The prevailing opinion that spinach, in spite of its high content of calcium, is a poor dietary source of this element, is not based upon a consistent array of evidence. The literature has been adequately reviewed in December, 1937, by the Council on Foods of the American Medical Association and need not be referred to here except with reference to oxalic acid metabolism. The presence of oxalic acid in spinach has been almost unanimously held responsible for the poor utilization of spinach calcium, revealed, indicated or only suggested by many experiments on laboratory animals and human subjects. But little work of a critical nature has been reported either on the availability of calcium oxalate in animal nutrition or upon the effect of the addition of soluble oxalates to a calcium-containing diet upon the availability of the contained calcium.

Spinach has been variously analyzed to contain 0.31% (Nelson and Mottern, '31), 0.29 to 0.69% (compilation by Kohman, '34) and 0.84% (Widmark and Ahldin, '33) oxalic acid on the fresh substance. The oxalates of spinach are absorbable since, although the normal oxalate content of the blood may not be raised by the consumption of spinach and other oxalate-containing vegetables (Athanasion and Reinwein, '34), the normal oxalate excretion in the urine is thus

[1] This investigation was made possible by the donation of funds to the University of Illinois by the American Dry Milk Institute.

increased several hundred per cent in both rabbits (Herkel and Koch, '36) and in men (Eimer and Bartels, '32). Possibly the occurrence of saponin in spinach, with its reputed favorable effect on the absorption of salts (Kofler, '27, '31), may account in part for such absorption.

To what extent calcium oxalate is available for body purposes and to what extent soluble oxalates impair the utilization of calcium in other forms, is not known definitely, although both questions have been the subject of experimental study. The calcium of spinach was found to be soluble to the extent of 30% on digestion of the dried material in pepsin-HCl solution by Horwitt, Cowgill and Mendel ('36). Kohman and Sanborn ('35) in a preliminary note have reported that the availability of the calcium in calcium oxalate is "of a low order," although it "does supply some calcium for bone formation." On adding sodium oxalate to diets containing practically all of their calcium in the form of skim milk or of dried kale so that the ratio of oxalic acid to calcium was the same as that in spinach, Fincke and Sherman ('35) observed depressions in calcium utilization of 38 and 24%, respectively. These results were obtained with diets containing less than sufficient calcium for normal calcification. With generous concentrations of calcium (0.61%), phosphorus (0.7%) and vitamin D (4 drops per kilogram of viosterol 250 D) in the diet, Mackenzie and McCollum ('37) found that the presence of 0.9% potassium oxalate in the diet did not affect growth or the ash content of the bones during 10 weeks of feeding. Even 2.5% of potassium oxalate did not affect growth, though the percentage ash in the bones was depressed. With borderline calcium (0.35%) and phosphorus (0.35%) in the diet and no vitamin D, even when the molarity of oxalate in the diet equaled that of calcium, some of the latter was still utilized as evidenced by growth, the ash content of the bones, and the calcium retention.

In continuing investigations of the availability of spinach calcium and of the calcium in calcium oxalate for animal growth, it was the purpose of the authors to apply more critical experimental procedures to a problem that has been solved,

apparently, in such diverse ways by methods of ad libitum feeding. The availability of the calcium of fresh 'home-cooked' and canned spinach has been compared with that of the calcium of skim milk powder, prepared by the spray process, while the availability of calcium oxalate has been determined by comparing the retention of calcium on a diet containing a liberal content of calcium mainly in that form, with that of a diet containing an equal concentration of calcium mainly as calcium gluconate.

PLAN OF EXPERIMENT

In the comparisons of dried skim milk with fresh, cooked and canned spinach in respect to the availability of the contained calcium the paired-feeding method was used. All pairs of rats were fed to the limit of appetite a low-calcium basal diet containing about 27% of dried, ether extracted whole egg, 10% of sucrose, 10% of lard, 7% of dried yeast, 2% of cod liver oil, 4% of a salt mixture [2] containing neither calcium nor phosphorus, and 41% of starch. Fractional variations from these figures possess no biological significance. This diet contained in various mixes an average of 3.60% of nitrogen, 4.10% ash, 0.144% of calcium and 0.393% of phosphorus. The calcium supplements were fed in amounts to provide for each pair of rats the same quantity of calcium equivalent to about 5 mg. per gram gain in body weight. This is about half the amount of calcium retained per gram of gain under conditions permitting maximum calcification. To equalize the gains of pair mates, the slower gaining rat in each pair was fed enough sucrose in addition to its equalized food to raise its body weight to that of its pair mate.

[2] Salt mixture:

potassium citrate. H_2O	88.00
potassium sulfate	8.90
potassium chloride	84.10
ferric citrate. $3\ H_2O$	6.97
potassium iodide	0.02
manganous sulfate. $2\ H_2O$	0.098
sodium fluoride	0.248
potassium aluminum sulfate. $24\ H_2O$	0.045
sodium chloride	38.90
magnesium chloride	202.30
Total	429.581

Each comparative test included eight pairs of rats, each pair being of the same sex and litter and approximately of the same body weight, which varied initially in the various pairs from 29 to 72 gm. At the same time that the pairs were selected on the basis of average body weights for 3 consecutive days, a third rat for each pair, of the same sex and litter and nearly the same in body weight, was sacrificed and analyzed for calcium and in some cases for phosphorus. The calcium content of these control rats was used in computing the initial calcium content of the experimental animals. The feeding periods generally extended over 5 or 6 weeks, or until

TABLE 1

The percentage chemical composition of the calcium containing foods tested

FOOD	WATER	NITROGEN	ETHER EXTRACT	CRUDE FIBER	ASH	CALCIUM	PHOSPHORUS
Fresh 'curly leaf' spinach	90.26	0.56	0.32	0.90	1.61	0.086	0.055
Cooked spinach [1]	90.48	0.59	0.57	...	...	0.110	....
Canned spinach [2]	....	...	...	...	0.57	0.056	....
Skim milk powder	2.39	5.59	0.57	...	7.84	1.268	1.022

[1] Prepared by cooking in boiling distilled water for 25 minutes. Water was then drained off and found to contain 8.6 mg. Ca and 170 mg. P for 4363 gm. fresh spinach. Arkansas spinach used.

[2] Locally bought. Labelled 'fancy California spinach.'

a gain of about 100 gm. had been made. The average daily gains of individual pairs varied from 1.7 to 4.0 gm. The final calcium contents of the rats were determined by analyzing the carcasses.

The samples of spinach used in the various tests and the sample of skim milk powder used in all tests were analyzed chemically more or less completely with the results shown in table 1.

The amounts of the various supplements that contained 5 mg. of calcium, fed per gram of gain, were: fresh spinach, 5.79 gm.; cooked spinach, 4.55 gm.; canned spinach, 8.87 gm., and skim milk powder, 0.394 gm.

The fresh and cooked spinach were analyzed for oxalic acid by the method of Nelson and Mottern ('31) except that the oxalate precipitated as the calcium salt was determined by titration with $KMnO_4$. The results obtained, expressed on the fresh basis were 0.22% for fresh spinach and 0.24% for cooked spinach.

In the final test to determine the utilization of the calcium of the oxalate salt two rations were prepared very similar to that above described except for the inclusion in the one case of calcium oxalate and in the other of calcium gluconate so that the rations contained somewhat more than 1% of calcium. The analysis of the rations follows:

	Nitrogen	*Ash*	*Calcium*	*Phosphorus*	*Gross energy per gram*
Calcium oxalate ration	3.17	5.73	1.24	0.43	4.42
Calcium gluconate ration	3.24	7.14	1.13	0.36	4.41

The high ash content of the gluconate diet was the result of an attempt to make allowance for the fact that oxalic acid is very incompletely oxidized by the body while gluconic acid is easily oxidized. Hence a small amount of $BaSO_4$ (1.6%) was included in the gluconate diet to equalize the energy value.

These diets were fed by the double reversal method to four pairs of rats, weighing initially from 60 to 90 gm. Pair mates received equal amounts of food at all times and in three balance periods the diet comparisons were of the following description: period 1, gluconate vs. oxalate; period 2, oxalate vs. gluconate; and period 3, gluconate vs. oxalate. The excreta were collected for 10 days and each collection period was preceded by a 4-day preliminary period in which the same diet was fed in the same amount. Fe_2O_3 was used as a feces marker at the beginning and end of each collection period.

The rats were confined in crystallizing dishes of Pyrex glass covered by a cylindrical cage of galvanized iron wire mesh. They rested on a wire grid of ½-inch mesh raised about 2 inches above the bottom of the dish.

All calcium analyses were made by some appropriate modification of the McCrudden method.

RESULTS OF THE EXPERIMENT

The results of the comparison of skim milk powder and fresh spinach, as they relate to calcium metabolism, are summarized in table 2. The eight control rats in this test contained on the average 2.86% ash, 0.70% calcium and 0.58% phosphorus.

It will be noted that in all pairs the rat fed a skim milk supplement exhibited the greater final calcium content and stored the greater amount of calcium in its body. On the average the spinach rat retained only 45 mg. of calcium for each 100 mg. stored by the milk rat. Of the milk-diet calcium fed, an average of 90% was retained in the body, while of the spinach-diet calcium fed only 40% was retained. The basal diet supplied 373 mg. of calcium, or 44% of the total calcium consumed, mainly contained in the dried egg. The average amount of stored calcium was 760 mg. for the rats on the milk supplement and 342 mg. for the rats on the spinach supplement. The latter value is 92% of the calcium consumed in the basal diet and quite probably was derived entirely from it.

Assuming that the utilization of the calcium in the basal diet was not differently affected by the different calcium supplements fed, the average difference in calcium storage of 418 mg. must have resulted from the difference in utilization of milk and spinach calcium, of each of which 472 mg. were consumed on the average. The two extreme interpretations of the difference in calcium storage under discussion are 1) the milk calcium was utilized to the extent of 100% and the spinach calcium to the extent of 11% $[(472 - 418) \div 472 \times 100]$, and 2) the milk calcium was utilized to the extent of 89% and the spinach calcium was not utilized at all. The latter interpretation seems nearer the truth.

In this experiment the phosphorus metabolism was studied in a manner similar to the calcium metabolism, but since the phosphorus intakes of paired rats were not equalized the results are not so strictly comparable. An average of 1300 mg. of phosphorus was consumed by the milk-supplement rats and 1220 mg. by the spinach-supplement rats. In all pairs, the

TABLE 2

The availability of calcium in dry milk solids and in fresh spinach

	PAIR 1 ♂		PAIR 2 ♂		PAIR 3 ♂		PAIR 4 ♂		
	Milk solids	Fresh spinach	Milk solids	Fresh spinach	Milk solids	Fresh spinach	Milk solids	Fresh spinach	
Final body weight, gm.	176	171	195	189	181	174	167	165	
Final body length, mm.	191	191	202	197	192	193	191	185	
Calcium in body, %	0.65	0.42	0.63	0.41	0.65	0.43	0.64	0.40	
Calcium in body, mg.	1157	714	1229	775	1268	722	1062	640	
Calcium ingested, mg.	812	812	886	886	886	886	872	872	
Calcium retained, mg.	714	278	786	289	854	353	764	315	
Calcium retained, %	88	34	89	33	96	40	88	36	
Calcium ingested in basal diet in per cent of total intake	44	44	43	43	43	43	43	43	

	PAIR 5 ♂		PAIR 6 ♂		PAIR 7 ♀		PAIR 8 ♂		AVERAGE	
	Milk solids	Fresh spinach	Milk solids	Fresh spinach	Milk solids	Fresh spinach	Milk solids	Fresh spinach	Milk solids	Fresh spinach
Final body weight, gm.	173	172	155	154	137	139	158	164	168	166
Final body length, mm.	191	188	194	184	183	181	189	189	192.6	188.5
Calcium in body, %	0.65	0.44	0.69	0.39	0.71	0.56	0.67	0.45	0.661	0.438
Calcium in body, mg.	1118	739	1056	566	966	717	1072	716	1116	699
Calcium ingested, mg.	830	830	821	822	766	767	888	888	845	845
Calcium retained, mg.	733	354	756	286	662	392	814	465	760	342
Calcium retained, %	88	43	92	35	86	51	92	52	90	40
Calcium ingested in basal diet in per cent of total intake	44	44	43	43	45	45	45	45	44	44

former rats stored the greater amounts of phosphorus (556 vs. 352 mg. on the average) and exhibited the greater percentages of phosphorus in their carcasses (0.512 vs. 0.406 on the average). On the milk-supplemented diet, the rats stored 43% of the phosphorus consumed on the average, while on the spinach-supplemented diet only an average of 29% of the consumed phosphorus was retained. For each 100 mg. of phosphorus stored by the milk-fed rats, 63 mg. were on the average stored by the spinach-fed rats.

The results with the cooked spinach and the canned spinach, obtained with eight pairs of rats in each case, were so similar

TABLE 3

Average results of the comparison of the availability of calcium in skim milk powder and in fresh spinach, cooked spinach and canned spinach. Each figure is an average for eight rats

	TEST 1		TEST 2		TEST 3	
	Milk solids	Fresh spinach	Milk solids	Cooked spinach	Milk solids	Canned spinach
Final body weight, gm.	168	166	128	125	131	129
Final body length, mm.	193	188	178	173	183	178
Calcium in body, %	0.66	0.44	0.70	0.49	0.67	0.42
Calcium in body, mg.	1116	699	861	563	746	511
Calcium ingested, mg.	845	845	604	604	604	604
Calcium retained, mg.	760	342	544	242	444	214
Calcium retained, %	90	40	91	40	74	40
Calcium ingested in basal diet in per cent of total intake	44	44	42	42	46	46

to those with fresh spinach that, to save space, only the averages from these tests are given in table 3. The differences shown by corresponding averages were shown consistently by each of the eight pairs except for body weights and body lengths. For better comparison of all results, the averages for the milk vs. fresh spinach test are included in the table.

The similarity of results is shown by the fact that for each 100 mg. stored on the milk-supplemented diet, 45 mg. were stored on the fresh spinach diet, 44.5 mg. on the cooked spinach diet, and 48 mg. on the canned spinach diet. In each of the tests, the calcium contained in the basal foods consumed was

TABLE 4

Calcium balances on rations containing calcium oxalate and calcium gluconate

	PAIR 1		PAIR 2		PAIR 3		PAIR 4		AVERAGES	
	Rat 1 ♂	Rat 2 ♂	Rat 3 ♂	Rat 4 ♂	Rat 5 ♂	Rat 6 ♂	Rat 7 ♂	Rat 8 ♂	4 rats	4 rats
Period 1										
Ca salt in ration	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate
Food consumed in 10 days, gm.	75	75	75	72.8	74	73.5	42	42	67	66
Total Ca intake, mg.	932	851	932	826	919	833	522	476	826	747
Ca intake from basal foods, mg.	144	179	144	173	142	175	80	100	128	157
Ca retained, mg.	60	262	34	189	76	230	12	81	46	191
Period 2										
Ca salt in ration	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate
Food consumed in 10 days, gm.	100	100	100	100	100	100	64	64	91	91
Total Ca intake, mg.	1134	1242	1134	1242	1134	1242	726	795	1032	1130
Ca intake from basal foods, mg.	238	192	238	192	238	192	152	123	217	175
Ca retained, mg.	366	98	332	109	345	86	209	38	313	83
Period 3										
Ca salt in ration	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate	Oxalate	Gluconate
Food consumed in 10 days, gm.	73	73	100	100	100	100	90	90	91	91
Total Ca intake, mg.	907	828	1242	1134	1242	1134	1118	1021	1127	1029
Ca intake from basal foods, mg.	140	174	192	238	192	238	173	214	174	216
Ca retained, mg.	138	307	107	366	78	436	140	334	116	361

greater in amount than the calcium retained on the spinach-supplemented diets, and sufficiently greater that the calcium thus stored may well have been derived entirely from the basal foods.

Although the body weights of the rats were more or less successfully equalized by giving sucrose to the slower growing pair mate, the body lengths were in the large majority of pairs greater for the milk-fed rat. This was true in nineteen of the twenty-four pairs, while in three pairs the body lengths were the same. This clear-cut outcome demonstrates that a greater rate of skeletal growth was promoted by the milk supplement than by the spinach supplement. This may or may not have been a result solely of a difference in calcium utilization of these supplements.

The significant results of the calcium balance studies of rations containing liberal amounts of calcium mainly derived either from calcium oxalate or from calcium gluconate, are summarized in table 4. Taking the retentions on the calcium gluconate diet as the maximum possible under the conditions of this experiment, the retentions of calcium on the oxalate diet were markedly submaximal. For all periods and all rats, the average retentions of calcium in 10 days were 288 mg. on the gluconate diet and only 82 mg. on the oxalate diet. The calcium intakes from the basal foods averaged 197 mg. in 10 days for the gluconate diet and 159 mg. for the oxalate diet. Thus, again the consumption of calcium in the basal foods was much greater than the retention of calcium on the oxalate diet and probably was the entire source of such retention.

CONCLUSIONS

The calcium of spinach, in either the fresh, cooked (open kettle), or canned form, is very poorly utilized for the maintenance and growth of rats; in all probability it is quite unavailable under conditions permitting a very high utilization of milk calcium.

Calcium oxalate appears to be entirely unutilizable by the growing rat.

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RADIOGRAPHIC DEMONSTRATION OF PROTECTION BY VITAMIN D AGAINST METAPHYSEAL DECALCIFICATION IN ADULT RATS ON HIGH CALCIUM—LOW PHOSPHORUS DIET

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FIVE FIGURES

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INTRODUCTION

A survey of the extensive literature dealing with the general problems of calcium and phosphorus metabolism fails to uncover more than a very limited amount of information concerning an interesting and important phase of this subject. That is, the role played by vitamin D in the normal mineral economy of the adult. One finds clinical opinion reflected in such statements as that of Peters and Van Slyke ('32):

Vitamin D is essential during infancy to prevent the development of rickets. For the fully grown animal, the amounts required are so small that no special care need usually be taken to provide them. In the adult, with normal serum calcium and phosphorus, vitamin D in moderate doses appears to have no demonstrable effect on calcium and phosphorus metabolism.

This attitude is based on studies similar to those of Hart, Tourtellotte, and Heyl ('28) who found that even on slightly acidotic diets of low calcium content, neither cod liver oil, nor ultraviolet irradiation had any beneficial effect on the calcium, phosphorus, or magnesium conservation in the adult human male.

When the situation is complicated by pregnancy and/or lactation, together with insufficient mineral intake, the picture is somewhat different. Here, the resulting osteomalacia responds to vitamin D therapy providing the mineral intake is made adequate, as shown by Maxwell and Miles ('25). The mineral balance in lactating animals such as the dairy cow, goat and rat, has been extensively studied. It is generally conceded that vitamin D is only one of a number of factors influencing the stability of the skeletal tissues under these conditions.

In view of these facts, it seemed of interest to determine to what extremes of mineral imbalance and vitamin D deprivation it is necessary to go to produce a symptomatology resembling adult osteomalacia in rats when the picture is uncomplicated by the cycle of reproduction. We also wished to determine whether vitamin D alone would have an effect on the mineral conservation while the diet was severely unbalanced with respect to calcium and phosphorus.

Accordingly, in the experimental series reported here, mature young albino rats were placed on the high calcium-low phosphorus diet of Steenbock and Black (no. 2965). The course of the experiment consisted of a prolonged depletion period on the basal diet only, followed by a shorter period during which one-half the animals were given a moderate maintenance dose (2.0 I.U. daily) of vitamin D. Since this basal ration is not adequate for prolonged maintenance due to insufficient vitamin B complex as well as being inadequate in vitamin A, these factors were added during the final 8 weeks of the experimental period in order to show that their presence in, or absence from the diet, has no effect on bone calcification.

The observation of these animals over a period of 1 year has demonstrated that even the rat's notorious resistance to derangement of its mineral economy is not proof against sufficiently long depletion periods. Furthermore, vitamin D is able to afford considerable protection against depletion of the skeletal stores, even when the mineral intake is far from optimum.

We were especially interested in being able to demonstrate these effects radiographically by means of a simplified technique to be described herewith.

EXPERIMENTAL

Radiographic technic. While a preliminary report of this procedure has already been published (O'Brien and Morgareidge, '34), subsequent use of the method over a period of several years has resulted in considerable refinement.

Fig. 1 General view of x-ray unit installed for small-animal radiography. The position of the falling-weight timer may be seen just behind the stool in the foreground.

Through the use of suitable protection for the operator, it is possible for the animals to be held manually during a short x-ray exposure, thus doing away with the necessity for either mechanical clamping or anesthesia, both of which procedures introduce physiological hazards which make repeated examinations of the animals difficult.

The general arrangement of the x-ray installation is shown in figure 1. The tube housing is covered by ⅛ inch of sheet lead and, directly beneath the cathode, carries a lead cone whose lower aperture projects to within 1 inch of the film holder and thus defines the dimensions of the x-ray beam. The method of holding the rat for radiography of the left knee joint is indicated in figure 2. The exposure is made posterior-anteriorly with a small degree of lateral rotation to bring the fibula slightly to one side. The film table, which just accommodates the dental film packet, has a lead top.

Fig. 2 Detailed view of a rat being held in position for radiograph of left knee joint.

Due to this fact, and to the small amount of rat tissue exposed to the beam, no appreciable back-scatter of radiation occurs. With a little practice, one operator, working unassisted, can radiograph between sixty to seventy rats per hour.

Using Eastman dental film, type A emulsion, and standard development technique (6 minutes at 65°F.), we find 1/15 second exposures give good quality films when the target-film distance is 16 inches and the current is 15 milliampere-seconds at a 60 kilovolt peak. The small size of the anatomical parts being studied makes imperative the achievement of the absolute maximum resolving power of the equipment. Kilovoltage

should be kept at a minimum without overloading the tube filament. The use of intensifying screens or high speed, coarse grained emulsions cannot be recommended.

The duration of the x-ray discharge is controlled by a falling weight timer inserted in the oil switch relay circuit. A heavy brass block, mounted between two vertical guide rods, carries a spring contact which bridges the gap between two brass rails during a fixed fraction of its free fall. The exposure time is thus determined by the length of the brass rails and is extremely constant since it is dependent only on the acceleration due to gravity. The block is released by a foot lever and is reset simply by lifting to the top position after each exposure. The contacts automatically disconnect during the resetting operation to eliminate the possibility of an accidental exposure.

For purposes of comparison, the films are mounted in a standard cardboard dental film holder which has been cut into strips of appropriate length.

Effects of high calcium—low phosphorus diet. Sixty albino rats, of about equal distribution between the sexes, were placed on the no. 2965 diet when approximately 19 weeks old and of an average weight of 195 gm. During the ensuing 34 weeks, the animals were given no supplement of any sort. Radiographs were made periodically of the lateral lumbar spine, tail, and left knee joint. Weight and clinical appearance records were kept. Weight continued to be gained slowly throughout the entire period and the animals maintained a remarkably good appearance in spite of the rigorousness of the diet. A few of the animals presented definite evidence of dietary deficiency. Several instances of sore eyes with sores around the gums and loose incisor teeth were noted. There were also a number of cases of a peculiar dermatitis consisting of pustular erruptions about the shoulders and along the sides of the neck and back which tended to form scabs. The ears, feet and tails remained normal and clean in appearance although the coats of the whole group were shaggy and unkempt and the hair had a chalky texture.

By the end of this 34-week period, the radiographs began to show evidence of marked decalcification of the skeleton, particularly in the proximal areas of the tibiae. There was noticeable thinning of the diaphyseal shaft and, surprisingly, the metaphysis developed a decalcified space approaching in appearance that of infantile rickets. Since it seemed beyond question that the animals were, by this time, seriously depleted of all vitamin D stores, they were split into two groups, one of which began to receive 2.0 international units of vitamin D daily in the form of irradiated ergosterol (Parke-Davis viosterol). The supplement was dissolved in corn oil and administered directly from calibrated dropping pipettes. The other group, serving as negative control, remained on the basal ration alone.

By the time this procedure had been in operation for 10 weeks (forty-fourth week of the experiment), it was seen that the physical condition of the rats in both groups was going downhill very rapidly. As is seen in figure 3, both groups had lost weight (those without vitamin D more rapidly). This was accompanied by a loss of appetite, increased severity of the dermatitis and death in a few instances. Therefore, in view of the known deficiency of the basal diet with respect to vitamin A and the B complex (especially flavins), 2% of whole dried yeast (Northwestern, unirradiated) was added to the diet and 10 U.S.P. units of vitamin A in the form of carotene (S.M.A. Corp.) were administered to each rat daily. The yeast was assayed for vitamin D potency and found to be entirely inactive in doses up to 800 mg. daily. As a result of this change of diet, the general condition improved rapidly. The eyes and mouths cleared up, appetites improved, and weight increases of as much as 30 or 40 gm. resulted in 2 weeks. The coats improved in appearance and, although it failed to disappear entirely, the dermatitis was noticeably improved.

After the addition of yeast and carotene to the diet of both groups, the experiment was continued for 8 additional weeks, making a total of 52 weeks covered by the study. In figure 3,

are plotted the average growth curves of the animals for the entire period. The solid line represents the average weight of the thirty rats which began to receive vitamin D at the point indicated; and the broken line represents the average weight of the negative controls.

Radiographic evidence. The results of the radiographic examinations are most striking in the proximal areas of the

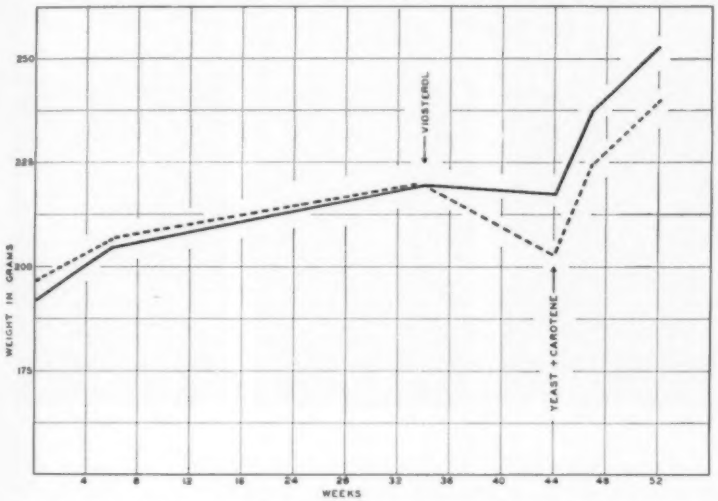
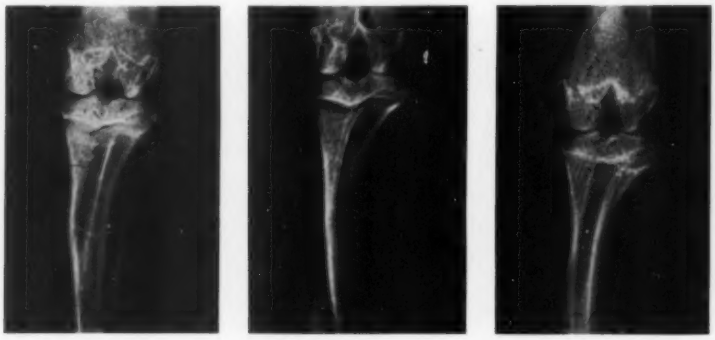


Fig. 3 The solid line represents the average growth of the thirty rats in the +D group. Administration of the vitamin (Viosterol) was begun at the point indicated. The dotted line represents the average growth of the control group. Yeast and carotene were added to the diets of both groups at the point indicated. The rats were 19 weeks old at the beginning of the experiment.

tibiae. This region, especially the metaphysis, appears to be one of the most sensitive to calcifying and decalcifying influences, even in the adult animal. The development of a typically rachitic metaphyseal zone of decalcification was perfectly definite in the —D group and the effectiveness of 2.0 I.U. of vitamin D in promoting recalcification in this area was equally clear. However this amount of vitamin D does not result in bones of entirely normal radiographic appearance

when given as a supplement to the Steenbock diet, even after adequate amounts of vitamins A and B have been added. Of the twenty-eight surviving rats in the +D group at the end of the 18-week period of vitamin D administration, there was evidence of incomplete recalcification of the metaphysis in five cases. Of the remaining twenty-three rats, although the metaphyses themselves were well filled in, the diaphyseal and epiphyseal shadows were less dense than at the beginning of the experiment 52 weeks previously.



a b c

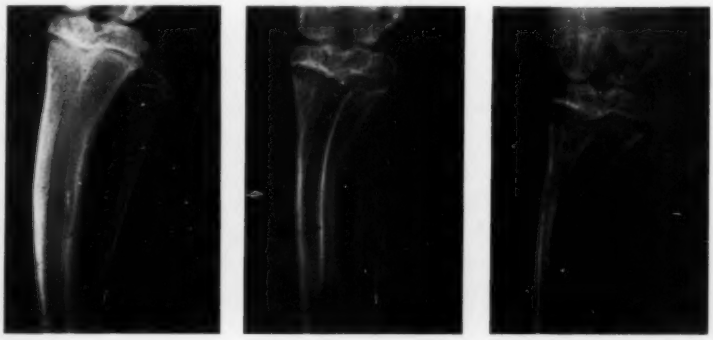
Fig. 4 Series of tibial radiographs of rat from +D group: a, at beginning of depletion period. b, after 34 weeks on Steenbock diet. c, after 2.0 I.U. vitamin D daily for 18 weeks.

Figure 4 shows reproductions at 3 diameters magnification of a set of radiographs from one rat of this group: 4 a gives the appearance of the bone at the beginning of the depletion period; 4 b shows its appearance after 34 weeks on the Steenbock diet; 4 c, taken at the end of the experiment and after administration of 2 units of vitamin D for 18 weeks, shows the disappearance of the metaphyseal space seen in 4 b. Note, however, the prominent trabeculation indicating subnormal calcification of the entire structure.

The rat illustrated in figure 5 is from the negative control group. Compare the results of 34 and 52 weeks without

vitamin D (5 b and 5 c) with the original normal structure (5 a). Twenty-eight animals survived in this group also, and the rat shown is typical of twenty-six of this number.

It should be said that the radiographs of the spines and tails failed to show a train of events at all comparable with that seen in the knee joint.



a b c

Fig. 5 Series of tibial radiographs of rat from —D group: a, at beginning of depletion period. b, after 34 weeks on the Steenbock diet. c, after 52 weeks on the Steenbock diet. Note increasing loss of bone density and development of metaphyseal space.

TABLE 1

Analyses of femurs

	Moist weight	*Dry defatted*	*Ash weight*	*Per cent of ash* Moist	Defatted	*Ca/P*
Males						
+ D	2.7842	1.7314	1.2915	46.4	74.6	2.22
— D	2.3573	1.3922	0.9931	42.1	71.3	2.24
Females						
+ D	2.3323	1.4523	1.0933	46.9	75.3	2.22
— D	2.3883	1.3321	0.9136	38.3	68.6	2.24

Bone-ash analysis. As a check on the radiographic evidence, two males and two females from each group were selected for femur ash determination and the ash from the four femurs pooled for calcium and phosphorus determinations. The inorganic ash, expressed as percentage of fresh, moist weight

was found to be 46.5% for both the males and females of the +D group. The males in the —D group had a femur ash of 42.1% and the females, 38.3%. The Ca : P ratio of the inorganic ash was found to be 2.23 ($\pm$0.01) for all the femurs analyzed. This constancy in the composition of bone ash under adverse dietary conditions is a common finding in spite of wide variations in the actual amount of ash. These analyses are summarized in table 1.

DISCUSSION

It appears to be beyond argument that the role played by vitamin D, even in moderate doses, is an important one in assisting the adult rat to maintain as nearly normal bone structure as possible in the face of an unbalanced mineral intake from the diet. This conclusion is in agreement with the findings of Kletzein, Templin, Steenbock and Thomas ('32). These workers maintained adult male and female rats on the no. 2965 diet for approximately 29 weeks and observed a reduction in femur ash of 10 and 9%, respectively. In a parallel series of animals whose diet had been directly irradiated with ultraviolet light to allow a daily intake of vitamin D of between 1 and 2 Steenbock units, it was found that loss of mineral elements from the skeleton was largely but not entirely prevented. They also point out that this basal ration is inadequate with respect to other vitamins than D as well as falling short in protein requirements for long maintenance experiments.

In this respect, it is interesting that the addition of the vitamin B complex and vitamin A during the final period of our experiment had no demonstrable effect on the final results insofar as recalcification in the depleted animals is concerned. In evaluating experiments of this kind, it should be remembered that the natural resistance of the rat to rickets on diets of normal calcium and phosphorus content has never been satisfactorily explained. Consequently, it is not the ideal test animal for studies designed to demonstrate the role of vitamin D in adult nutrition.

SUMMARY

Adult rats have been kept on the no. 2965 rachitogenic diet of Steenbock and Black for a period of 52 weeks. By means of a special radiographic technique, as well as by bone ash determinations, it was possible to show that considerable decalcification of the skeleton occurred. This condition was markedly improved, although not completely corrected, by moderate doses of vitamin D administered during the last 18 weeks of the experiment. It has also been shown that the structures of the proximal end of the tibia, even in the adult animal, are especially sensitive to changes in mineral metabolism which affect calcification. Adult rats deprived of vitamin D, develop a rather typical decalcified metaphyseal space which may be recalcified by 2.0 international units of vitamin D per day, even on this restricted basal ration. Addition of whole dried yeast and carotene improved the general physical condition of the animals but failed to result in any demonstrable change in the bones.

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The original plan of the experiment was laid out in collaboration with Dr. H. Douglas McEwen of the department of biochemistry who also supervised the care of the animals throughout the first stages of the experiment. The bone ash and calcium and phosphorus determinations were made by Dr. Harold C. Hodge and Miss Marian LeFevre.

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